IMMUNOLOGICAL AND BIOSYNTHETIC STUDIES ON THE MAMMALIAN 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

A thesis submitted for the degree of DOCTOR OF PHILOSOPHY

by

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ABBREVIATIONS

In addition to the accepted abbreviations (Instructions to Authors, Biochem. J. (1985) 225, 1-26), the following have been adopted:

BCDC	branched chain 2-oxo acid dehydrogenase complex
bp	base pairs
BRL	buffalo rat liver cells
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CHO	chinese hamster ovary cells
DMSO	dimethylsulphoxide
2,4-DNP	2,4-dinitrophenol
DOC	deoxycholate, sodium salt
DTNB	5,5'-dithiobis[2-nitrobenzoic acid]
DTT	dithiothreitol
FCCP	carbonyl cyanide p-trifluoromethoxy phenyl hydrazone
Iodogen	1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril
leupeptin	acetyl-L-leucyl-L-leucyl-L-argininal
LMM	low methionine medium
MMM	minus methionine medium
NBL-1	bovine kidney cells
NEM	N-ethylmaleimide
NGM	normal growth medium
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDC	pyruvate dehydrogenase complex

PEG	polyethylene glycol
PK-15	porcine kidney cells
PMSF	phenylmethylsulphonylfluoride
PPO	2,5-diphenyloxazole
RBS	rat blood serum
SDS	sodium dodecylsulphate
SRP	signal recognition particle
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPCK	L-1-tosylamide-2-phenylethylchloromethyl ketone
TPP	thiamine pyrophosphate
Tween 20	polyoxyethylenesorbitan monolaureate

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SUMMARY

High, titre monospecific polyclonal antisera have been raised against purified mitochondrial 2-oxoglutarate dehydrogenase complex (OGDC) and its component enzymes, 2-oxoglutarate dehydrogenase (E1), lipoyl succinyltransferase (E2) and lipoamide dehydrogenase (E3). These specific antisera have been employed to monitor molecular events in the biosynthesis, import and maturation of this multimeric assembly. In antiserum raised against native, intact 2-oxoglutarate dehydrogenase complex, lipoamide dehydrogenase elicits a poor antibody response in comparison to the other polypeptides of the complex. It is thought this may reflect the conserved nature of lipoamide dehydrogenase which is involved in highly differing subunit interactions with the distinctive lipoyl acyltransferase (E2) 'core' enzymes of each of the individual 2-oxo acid dehydrogenase complexes.

In cultured porcine kidney (PK-15) and bovine kidney (NBL-1) cells, incubated with [^{35}S]methionine in the presence of uncouplers of oxidative phosphorylation, appearance of higher M_r forms of the individual enzymes can be detected by specific immune precipitation and fluorographic analysis. In the cases of 2-oxoglutarate dehydrogenase, E1, and lipoamide dehydrogenase, E3, the initial translation products have subunit M_r values 1000-3000 greater than the mature enzyme while the precursor of lipoyl succinyltransferase, E2, contains an additional sequence of M_r 6000-8000. Competition studies have revealed the immunological similarity of the precursor molecules to the native subunits. The precursor form of lipoyl succinyltransferase (pre-E2) is located in the cytoplasm of the cell

prior to its conversion to the mature form and import into mitochondria. These precursor molecules are relatively stable, remaining in the cell for several hours if the cells are maintained in the presence of uncouplers. On removal of uncouplers, processing is rapidly initiated and is complete within 40 min. Interestingly antiserum to native 2-oxoglutarate dehydrogenase complex fails to recognise E2 precursor molecules which can be immunoprecipitated, however, by antibodies raised against the denatured E2 subunit. It is concluded that pre-E2 is conformationally dissimilar to native E2 which exists normally as a highly-ordered, multimolecular aggregate in the native complex.

CHAPTER ONE

INTRODUCTION

1.1 PROTEIN SORTING

Eukaryotic cells exhibit a high degree of organisational complexity. Intracellular membranes divide the cells, physically and biochemically, into several discrete compartments, each of which performs a different specialised function. This function is reflected in the unique protein composition of each organelle. Most cellular polypeptides are located exclusively in one particular compartment.

Since the vast majority of protein synthesis occurs in the cytoplasm, biogenesis of protein is therefore often associated with passage of newly-synthesised proteins through biological membranes. This is seen in the case of integral membrane proteins, secreted proteins and proteins bound for the intracellular organelles.

In the cell there is a requirement for these newly-synthesised proteins to be sorted from each other and routed for export, or targetted to the correct intracellular membrane or compartment. One of the simplest hypotheses proposed to explain this predicts that each protein contains a specific sequence or structure which is recognised by a complementary structure on the membrane to which it is targetted. The repertoire of these distinct "topogenic sequences", constituting a permanent or transient part of the polypeptide chain, is predicted to be relatively small as many different proteins will be bound for the same intracellular site (Blobel, 1980).

Intracellular membranes must have the ability not only to recognise proteins specifically destined for the compartment which they delineate, but also the capacity to translocate these proteins across the lipid bilayer, a remarkable feat when the size and hydrophilic nature of many of these proteins is taken into account.

The precise nature of the information required and events involved in directing proteins to a particular location have been subject to intense study over the past fifteen years.

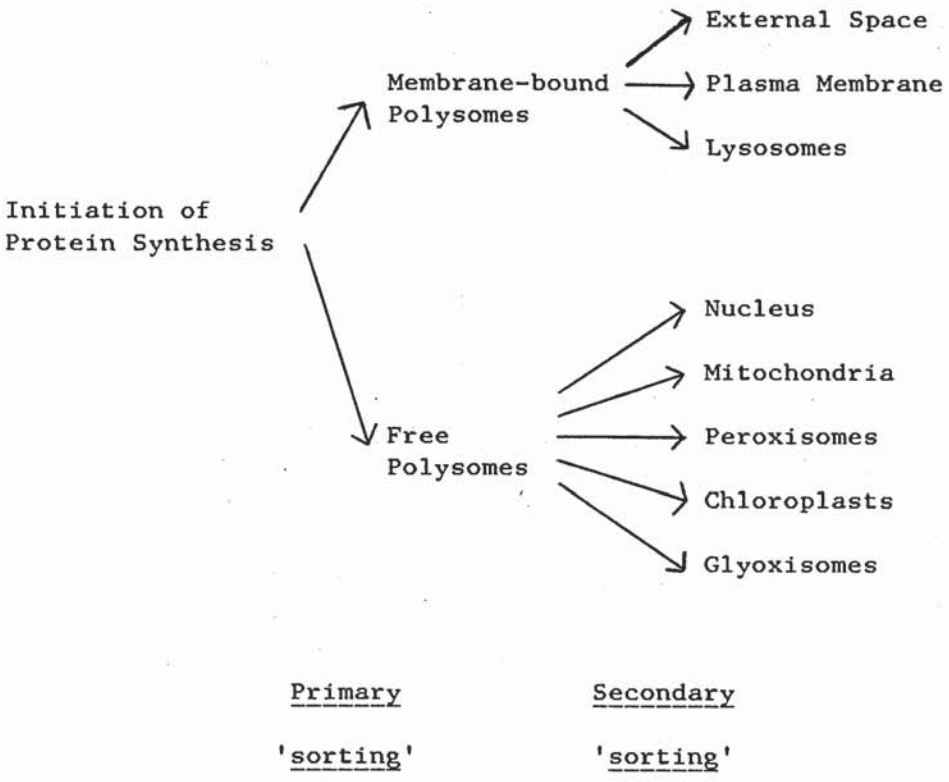
1.2 AN OVERVIEW OF MEMBRANE AND ORGANELLE ASSEMBLY

The mechanisms involved in unidirectional transport of proteins across membranes and their insertion into membranes may be divided into two classes of process, cotranslational and posttranslational (Sabatini et al., 1982). In the case of proteins traversing the membrane of the endoplasmic reticulum the mechanism generally employed is cotranslational, i.e. vectorial transport across the membrane is concomitant with protein synthesis. A contrasting scheme applies to proteins destined for the nucleus (De Robertis et al., 1978), peroxisomes (Goldman & Blobel, 1978), glyoxisomes (Zimmerman & Neupert, 1980b), chloroplasts (Chua & Schimdt, 1978; Highfield & Ellis, 1978) and mitochondria (Neupert & Schatz, 1981). This alternative mechanism is a posttranslational process in which translocation is independent of protein synthesis.

1.2.1 COTRANSLATIONAL TRANSPORT

Many proteins are translated on ribosomes tightly-associated with the endoplasmic reticulum. They are vectorially discharged through the membrane during the elongation phase of protein synthesis. Completed polypeptides belonging to this class of proteins are never seen on the cytosolic side of the membrane which binds the ribosomes. For many of these proteins transfer across the endoplasmic reticulum is the first step in a complicated process of transfer to locations such as the

FIGURE 1.1 General scheme for distribution of proteins in eukaryotes.



lysosomes, golgi apparatus, plasma membrane or to secretory granules for eventual release from the cell (see Fig.1.1).

Blobel & Dobberstein, (1975) first proposed, in the 'signal hypothesis', a detailed mechanism describing how cotranslationally transported proteins could reach their correct initial location i.e. the endoplasmic reticulum. This hypothesis has been extensively modified since then and to date entails the following steps: Polypeptide synthesis is initiated on free ribosomes. A 'signal sequence' of 15-30 amino acids in length, located at the NH_2-terminus of the nascent polypeptide chain (Milstein et al., 1972), protrudes from the ribosome once a polypeptide of approx. 60-70 amino acids in length has been synthesised. An oligomeric 'signal recognition particle' (SRP), containing 6 proteins, ranging in M_r from 9000-72000 (Walter & Blobel, 1980), and a 7S RNA molecule (Walter & Blobel, 1982), then binds to the freely accessible 'signal sequence' and arrests further elongation of the polypeptide chain (Walter et al., 1981). Interaction of the SRP-polysome complex with the cytoplasmic domain of a 72000 M_r 'docking protein' (Meyer et al., 1982a), located in the endoplasmic reticulum membrane releases the arrest of elongation (Meyer et al., 1982b), and translation coupled to translocation proceeds. Ribosome-membrane interactions may be further stabilised by binding of the large ribosomal subunit to other proteins of the endoplasmic reticulum, such as the ribophorins (Kreibich et al., 1978a,b). Cleavage of the 'signal peptide', at a specific amino-acyl bond, usually before the polypeptide chain is completed, is accomplished by a specific integral membrane endoproteinase, known as 'signal peptidase', located at the luminal side of the endoplasmic

reticulum membrane (Jackson & Blobel, 1977).

This sequence of events ensures efficient, specific translocation of proteins across the endoplasmic reticulum and guards against an inappropriate accumulation of these proteins in the cytoplasm.

1.2.2 POSTTRANSLATIONAL TRANSPORT

In contrast to the above scheme posttranslationally imported proteins are synthesised on free polysomes. Completed polypeptide chains are released into the cytosol to form pools of free precursor which are rapidly depleted by uptake of proteins into the organelles. Precursor polypeptides are distinct from their mature counterparts either in size, charge or conformation. A common feature of precursor proteins is the presence of an NH_2-terminal extension sequence. Specific receptor molecules,located on the outer surface of the organelles, are proposed to mediate direct interaction of precursors with their target membranes. Processing of the precursors to their mature form accompanies translocation across the membrane. Removal of the amino-terminal extension sequences of larger precursors is effected by a specific endoprotease. Conformational changes associated with the processing event are thought to result in the mature protein being trapped in the membrane or inside the organelle. Synthesis of the polypeptides and subsequent transport and processing are evidently separate events (Sabatini et al., 1982).

1.2.3 ASSEMBLY OF MITOCHONDRIA DEPENDS ON TWO GENETIC SYSTEMS

The posttranslational import mechanism described above applies to nuclei, peroxisomes, glyoxisomes, chloroplasts and mitochondria (see Fig.1.1). However the biogenesis of chloroplasts and mitochondria is further complicated in that it requires contributions from more than one genetic system: the nuclear system and a second system located in the organelle itself (Schatz & Mason ,1974). These genetic systems are physically separate and there is no transfer of mRNA between the two compartments. Molecules derived from both genomes have received so much attention in the past few years that each system now constitutes a separate area of research.

Mitochondrial DNA is generally circular and varies considerably in size between species from 1.4×10^4bp in animal cells to 2.4×10^6bp in some higher plants (de Vries & van't Sant, 1983). The nature of the genetic information encoded in mitochondrial DNA has gradually become apparent in the last fifteen years and is roughly the same in all cases studied. Complete sequence analysis of a number of mitochondrial DNAs has been performed, the human mitochondrial DNA sequence being first to be elucidated (Anderson *et al.*, 1981). Transcription products of mitochondrial DNA include two rRNA species, all tRNAs required for mitochondrial protein synthesis, and mRNAs coding for a limited number of proteins. Proteins encoded by mitochondrial DNA are mainly hydrophobic subunits of the respiratory complexes of the inner mitochondrial membrane: cytochrome bc_1, cytochrome c oxidase and the mitochondrial ATPase. In *Saccharomyces cerevisiae* (hereafter referred to as yeast) and *Neurospora crassa*, an

additional ribosome-associated protein is encoded in the mitochondrial DNA (Terpstra & Butow, 1979; LaPolla & Lambowitz, 1981). However in a study performed in a bovine kidney cell line, Schieber & O'Brien (1985) found that all mitochondrial ribosomal proteins were synthesised in the cytosol and encoded in nuclear DNA. Sequence analysis of human mitochondrial DNA revealed the presence of several unidentified reading frames (URF) potentially coding for protein, raising the number of possible translation products in the human system to thirteen. A recent report has suggested that six of these URFs code for proteins of the NADH dehydrogenase complex (Chomyn et al., 1985).

Considering the total number of mitochondrial proteins, approximately 95% are synthesised in the cytoplasm and imported into mitochondria posttranslationally.

1.2.4 MITOCHONDRIAL PROTEINS ARE FOUND IN ONE OF FOUR LOCATIONS

Mitochondria consist of two membranes which differ in chemical composition, permeability and enzyme content. These two membranes divide the mitochondria into two different compartments: the intermembrane space and the matrix. This means that mitochondrial proteins must be routed to one of four locations: the inner membrane, the outer membrane, the intermembrane space or the matrix. Each of these compartments is unique with respect to its protein composition.

In comparison to other cellular membranes an unusual permeability is displayed by the outer mitochondrial membrane. This is due to the large channels, formed by porin, the most abundant protein in the outer membrane, which render the membrane permeable to molecules of M_r

< 6000. Relatively few other proteins have been identified in this membrane.

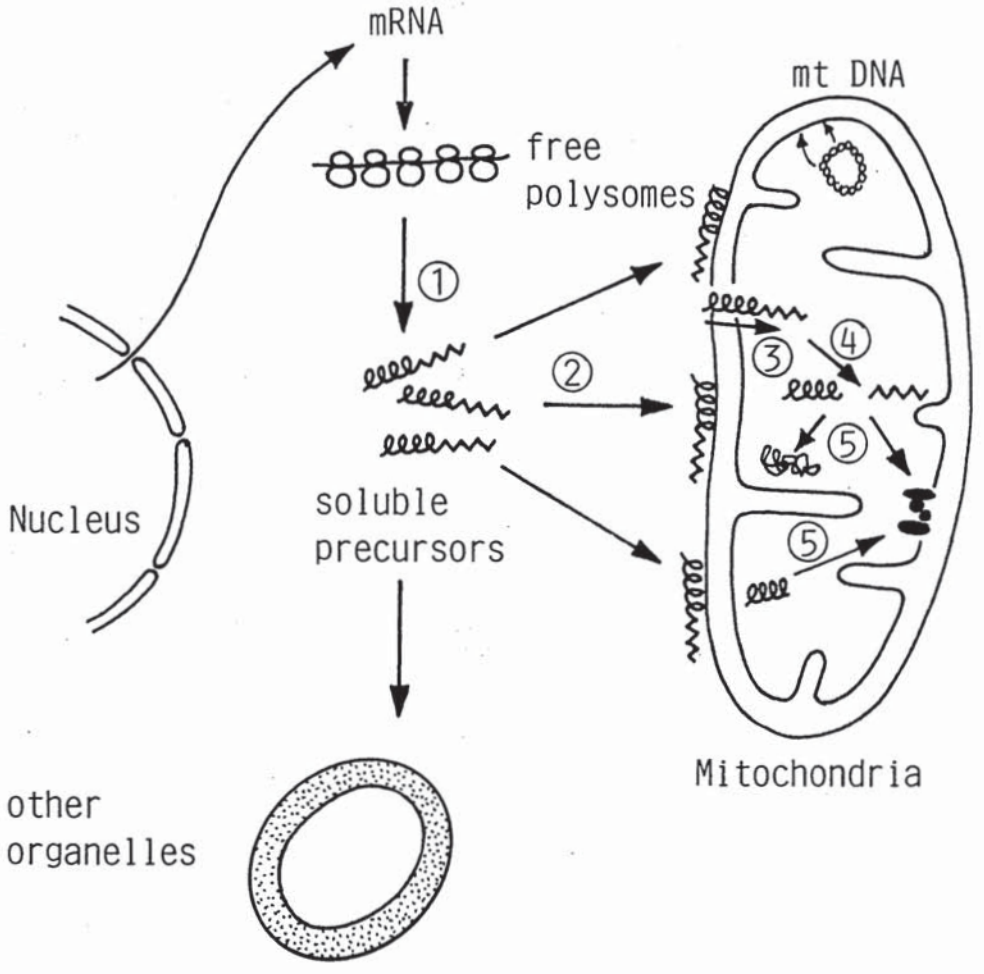
The inner membrane has a high protein content, displays selective permeability and contains a number of carrier systems, allowing the passage of low M_r metabolites required by the enzymes of the matrix. Extensive invagination of the inner membrane provides a large surface area for enzymes, and components of cellular respiration and oxidative phosphorylation.

Soluble enzymes of the inter-membrane space include adenylate kinase, sulphite oxidase (rat liver) and cytochrome b_2 (yeast). The latter two interact with cytochrome c, also located in the inter-membrane space but bound loosely to the inner membrane, where it mediates electron transfer between the cytochrome bc_1 and cytochrome c oxidase complexes.

The matrix of the mitochondrion contains a large number of soluble and hydrophilic enzymes, in particular those of the citric acid cycle. Replication and transcription of mitochondrial DNA and intra-mitochondrial protein synthesis also take place in this compartment.

Each membrane should present a barrier to the passage of most proteins. Therefore the transfer of several hundred mitochondrial proteins across these membranes to the various mitochondrial subcompartments is a complicated process. Experiments conducted in several laboratories over the past 5-6 years have led to a basic understanding of how mitochondria integrate the proteins synthesised in the cytosol.

FIGURE 1.2 Biogenesis of mitochondria



1. Precursor synthesis.
2. Recognition and binding to mitochondria.
3. Translocation.
4. Processing.
5. Assembly.

Incorporation of cytoplasmically-synthesised proteins into mitochondria can be conceptually divided into 5 stages (see Fig.1.2):

1. synthesis of the precursor polypeptide
2. recognition and binding of the precursor to the mitochondrial surface
3. translocation of the precursor across one or two mitochondrial membranes
4. processing of the precursor to the mature protein
5. assembly of the subunits into functional holoenzymes.

1.3 SYNTHESIS AND POSTTRANSLATIONAL TRANSPORT OF MITOCHONDRIAL PROTEINS

The two experimental approaches which have been used to study synthesis of mitochondrial precursors and their translocation into the organelle have employed highly specific antiserum raised against the mature protein with the assumption that cross-reaction with the precursor will occur. In the majority of cases studied to date this assumption has proved valid.

Cell-free translation systems allow the analysis of precursors as they are released from the ribosome. In vitro translation systems most commonly used are reticulocyte lysates and wheat germ extracts, both of which can be efficiently programmed with mRNA from any eukaryotic source. Translation is performed in the cell-free system, programmed with suitable mRNA, in the presence of [^{35}S]methionine or [^{14}C]leucine. The radioactive products are then treated with detergents, usually Triton X-100 or SDS, and incubated with highly specific antiserum raised against the protein of interest. Since

mitochondrial proteins are present in the translation mixture in such small amounts (0.02-0.5%) indirect immune precipitation is required, generally employing formalinised Staphylococcus aureus cells or protein A from Staphylococcus aureus coupled onto a solid support, such as Sepharose beads. Immune precipitated proteins are then resolved by SDS-polyacrylamide gel electrophoresis, visualised by fluorography and should be directly compared with radiolabelled, mature protein.

Translocation can be studied in this system by incubating isolated intact mitochondria with precursor proteins synthesised in vitro. Mitochondria can then be removed by centrifugation, solubilised and subjected to immune precipitation as described above. In order to establish that the precursor has been imported across the mitochondrial membrane(s) it must be shown that it has reached a protease-resistant location.

The second approach is based on experiments performed in vivo. Cells are subjected to 'pulse' or 'pulse-chase' labelling with [^{35}S]methionine or [^{14}C]leucine. Detergent extracts of the cells are employed for immune precipitation, SDS-PAGE and fluorography as previously described. Difficulties inherent in this second approach include low incorporation of radio-labelled amino acid into protein during the pulse, and the short half-life of precursor proteins in the extra-mitochondrial pool. An alternative procedure involves radio-labelling whole cells under conditions which prevent import of precursors into mitochondria, such as in the presence of uncouplers of oxidative phosphorylation (see section 1.5.1). The use of such a procedure is validated by the fact that the accumulated precursors can be chased into the mitochondria by reversing the effect of the uncouplers.

TABLE 1.1 (cont'd)

PROTEIN LOCATION	PROTEIN	ORGANISM	APPARENT M_r ($\times 10^{-3}$)		REFERENCE
			MATURE	PRECURSOR	
Inner Membrane	Cytochrome c_1	Yeast	31	37	Ohashi *et al.* (1982)
	Cytochrome bc_1 complex:	*N. crassa*			Teintze *et al.* (1982)
	subunit I	*N. crassa*	50	51.5	"
	subunit II	*N. crassa*	45	47.5	"
	subunit V	*N. crassa*	25	28	"
	subunit VI	*N. crassa*	14	14	"
	subunit VII	*N. crassa*	11.5	12	"
	subunit VIII	*N. crassa*	11.2	11.6	"
	Cytochrome c_1	*N. crassa*	31	38	"
Intermembrane Space	Adenylate kinase	Chicken	28	28	Watanabe & Kubo (1982)
	Cytochrome c	*N. crassa*	12	12	Korb & Neupert (1978)
		Rat	12	12	Matsuura *et al.* (1981)
	Cytochrome c peroxidase	Yeast	33.5	39.5	Maccecchini *et al.* (1979b)
	Cytochrome b_2	Yeast	58	68	Daum *et al.* (1982)
	Sulphite oxidase	Rat	55	59	Mihara *et al.* (1982b)
Outer Membrane	Monoamine oxidase	Rat	59	59	Sagara & Ito (1982)
	Porin	Yeast	29	29	Gasser & Schatz (1983)
		N. crassa	31	31	Freitag *et al.* (1982a,b)
	14000 M_r protein	Yeast	14	14	Hay *et al.* (1984)
	45000 M_r protein	Yeast	45	45	"
	70000 M_r protein	Yeast	70	70	"
	OMM-35	Rat	35	~35.5	Shore *et al.* (1981)

TABLE 1.1 (cont'd)

PROTEIN LOCATION	PROTEIN	ORGANISM	APPARENT M_r ($\times 10^{-3}$) MATURE	PRECURSOR	REFERENCE
Matrix	Phenylalanyl tRNA synthetase	Yeast			Diatewa & Stahl (1981)
	α-subunit		72	74	"
	β-subunit		57	61	"
	Propionyl-CoA carboxylase	Rat			Hay et al. (1984)
	α-subunit		70	74.5	"
	β-subunit		54	61.5	"
	RNA polymerase	Yeast	45	47	Lustig et al. (1982)
Inner Membrane	ADP/ATP carrier	N. crassa	32	32	Zimmermann & Neupert (1980a)
		Rat	30	30	Hatalova & Kolarov (1983)
	Subunit IX ATPase	N. crassa	8.2	14	Michael et al. (1979)
	Carnitine acetyltransferase	Rat	67.5	69	Miyazawa et al. (1983)
	Creatine kinase	Dog	42	48	Perryman et al. (1983)
	Cholesterol-side-chain-cleavage cytochrome P-450	Cattle	49	54.5	DuBois et al. (1981)
	Cytochrome c oxidase: subunit IV	Yeast	14	17	Lewin et al. (1980);
	subunit V	Yeast	12.5	15	Mihara & Blobel (1980)
	subunit VI	Yeast	12.5	17-20	"
	subunit VII	Yeast	5-7.5	5-7.5	"
	subunit IV	Rat	16.5	18-19.5	Schmelzer & Heinrich (1980)
	subunit V	Rat	12.5	15.5	"
	D-β-Hydroxybutyrate dehydrogenase	Rat	32	37	Mihara et al. (1982b)
	Cytochrome bc_1 complex:				Cote et al. (1979)
	subunit I	Yeast	44	44.5	"
	subunit II	Yeast	40	40.5	"
	subunit V	Yeast	25	27	"
	subunit VI	Yeast	17	25	"
	subunit VII	Yeast	14	14	"
	subunit VIII	Yeast	11	11	"

TABLE 1.1 Cytoplasmically synthesised mitochondrial proteins

PROTEIN LOCATION	PROTEIN	ORGANISM	APPARENT M_r ($\times 10^{-3}$)		REFERENCE
			MATURE	PRECURSOR	
Matrix	Acetoacetyl-CoA thiolase	Rat	38	41	Hay et al. (1984)
	Acyl-CoA dehydrogenase				
	general	Rat	39	41	"
	short-chain	Rat	36.5	41	"
	Adrenodoxin	Cattle	12	20	Nabi & Omura (1980)
	Adrenodoxin reductase	Cattle	~50	~50	"
	δ-Aminolevulinate synthase	Rat	45	51	Yamauchi et al. (1980)
		Chicken	63-65	75	Ades & Harpe (1981)
	Aspartate aminotransferase	Chicken	44.5	47	Sonderegger et al. (1980)
		Rat	45	47	Sakakibara et al. (1980)
	F_1-ATPase: α-subunit	Yeast	58	64	Maccecchini et al. (1979a)
	β-subunit	Yeast	54	56	"
	γ-subunit	Yeast	34	40	"
	Carbamyl-phosphate synthetase	Rat	160	165	Mori et al. (1979)
		Frog	160	160	Campbell et al. (1982)
	Citrate synthase	Yeast	47	50	Bohni et al. (1983)
		N. crassa	45	47	Harmey & Neupert (1979)
	Enoyl-CoA hydratase	Rat	25	29.5	Hay et al. (1984)
	L-Glutamate dehydrogenase	Rat	54	60	Mihara et al. (1982b)
	3-Hydroxyacyl-CoA dehydrogenase	Rat	29.5	33	Hay et al. (1984)
	2-Isopropylmalate synthase	Yeast	65	65	Gasser et al. (1982a)
	3-Ketoacyl-CoA thiolase	Rat	38	38	Hay et al. (1984)
	Malate dehydrogenase	Rat	37	38	Mihara et al. (1982b)
	Manganese superoxide dismutase	Yeast	24	26	Autor (1982)
	Methylmalonyl-CoA mutase	Rat	75	80.5	Hay et al. (1984)
	Ornithine aminotransferase	Rat	43	49	Mueckler et al. (1982)
	Ornithine transcarbamlyase	Rat	36-39	39.5-43	Mori et al. (1981a)
		Mouse	37	39.5	Hay et al. (1984)

This whole cell approach, despite its greater technical difficulty, offers the advantage of studying posttranslational import *in vivo*, thus ascertaining the validity of the results obtained *in vitro* as applied to the physiological situation.

The extreme sensitivity of these immunological techniques has been indispensable in the studies of biogenesis of mitochondria and other organelles.

1.3.1 MITOCHONDRIAL PROTEINS ARE SYNTHESISED AS LARGER PRECURSORS

In a report incorporating both of the approaches outlined above, Maccecchini *et al*. (1979a) revealed that the three largest, nuclear-encoded, subunits of the mitochondrial ATPase were initially synthesised as larger M_r precursors. These precursors were transferred *in vitro* into isolated mitochondria and cleaved to the corresponding mature forms in the absence of protein synthesis.

These results were similar to those already obtained in chloroplasts in a study involving the small subunit of ribulose-1,5-bisphosphate carboxylase (Chua & Schmidt, 1978; Highfield & Ellis, 1978).

In recent years evidence has accumulated for the existence of larger M_r precursors to many mitochondrial proteins (see Table 1.1). As can be seen from the table there are precursors to proteins of all mitochondrial compartments.

The difference in M_r between precursors and their mature counterparts varies between 500 and 10000, a difference generally greater than that observed in the case of secretory proteins.

In a few cases this difference in size has been shown to be directly due to an extra sequence of amino acids at the NH_2-terminus of the polypeptide. Comparison of the protein sequence of mature subunit IX of the N. crassa mitochondrial ATPase, with that of the precursor, deduced from the nucleotide sequence of the cloned gene, reveals an NH_2-terminal extension of 66 amino acids (Viebrock et al., 1982). A presequence consisting of 68 amino acids has also been predicted, from the gene sequence, for yeast cytochrome c peroxidase (Kaput et al., 1982). Primary translation products of the yeast F_1-ATPase α, β, and γ subunits, and subunits IV to VI of the cytochrome c oxidase complex have been labelled specifically at the NH_2-terminus using N-formyl-[^{35}S]methionine (Lewin et al.,1980; Mihara & Blobel, 1980). Since eukaryotic cells lack the methionine amino-peptidase required to remove this N-formyl-methionine, the fact that it is absent from the imported mature protein suggests the removal of a sequence from the NH_2-terminus of the precursor.

Specific labelling of the cytochrome c oxidase subunits with this reagent also refutes earlier claims that subunits IV - VII are synthesised as a polyprotein (Poyton & McKemmie, 1979).

There appears to be no correlation between the size of these extension sequences and the location of proteins in the mitochondrion, neither do precursors to subunits of the same multi-protein complex have a standard number of additional amino acids (Teintze et al., 1982).

In a few cases, comparison of precursor and mature forms does not reveal a difference in M_r. Proteins which do not have larger M_r precursors include cytochrome c, located in the inter-membrane space (Korb & Neupert, 1978), isopropylmalate synthase, a matrix enzyme

(Gasser et al., 1982a), the inner membrane ADP/ATP carrier (Zimmermann et al., 1979b; Hatalova & Kolarov, 1983) and porin, a major component of the outer membrane (Freitag et al., 1982b; Gasser & Schatz, 1983). For cytochrome c and the ADP/ATP carrier it has been demonstrated that import occurs without removal of NH_2-terminal amino acids by specifically labelling the protein with N-formyl-[^{35}S]methionine and showing that the mature, imported form still retains this label (Zimmermann et al., 1979a,b). Proteolytic cleavage therefore is not an obligatory step in protein transport.

The presence of highly hydrophobic membrane proteins in the cytosol, en route to mitochondria, indicates possible conformational differences between the precursor and mature forms. In the case of subunit IX of the ATPase complex, which is highly hydrophobic, the 66 amino acid presequence, already mentioned, is very polar and may function to maintain the protein in a soluble form. Conformational dissimilarities between mature and precursor proteins have been demonstrated for the precursor of the ADP/ATP carrier which binds hydroxylapatite where the mature form does not (Zimmermann & Neupert, 1980a) and the precursor form of the matrix enzyme fumarase, which differs from the mature form in that it does not bind to the pyromellitic acid-Sepharose 4B affinity column (Ono et al., 1985). Another indication of differences in tertiary structure is revealed in differential protease sensitivities of the mature and precursor forms of porin (Gasser & Schatz, 1983) and aspartate aminotransferase (Jaussi et al., 1982). In a minority of cases, a conformational difference is inferred when antiserum raised against the mature protein fails to recognise the precursor (Schmidt et al., 1983b). The

best example of this is in the case of cytochrome c of N. crassa. The apocytochrome c has the same M_r as the holoenzyme but lacks the covalently attached haem group. Antibodies raised against the holoenzyme fail to recognise the apocytochrome and vice versa (Hennig & Neupert, 1981). Obviously the haem group governs the different folding of the polypeptide chain in the two forms of the cytochrome and covalent attachment of the haem induces a strong conformational change in the protein.

Differences in tertiary structure are further suggested by demonstrating that precursor proteins, accumulated in the cytosol, tend to form aggregates. The precursor to fumarase (M_r 45000) migrates with an apparent M_r of 270000-360000 on gel filtration (Ono et al., 1985) and the in vitro synthesised ADP/ATP carrier (M_r 32000) forms aggregates of M_r 120000-500000 (Zimmermann & Neupert, 1980a). Sucrose density gradient analysis shows an apparent M_r of 80000-240000 for the precursor to subunit IX of the ATPase complex of N. crassa (Schmidt et al., 1983b). Other precursor proteins existing in aggregated states include the β-subunit of yeast F_1-ATPase and glutamic oxaloacetic transaminase, carbamyl phosphate synthetase and ornithine carbamyltransferase of rat liver (Miura et al., 1981; Sakakibara et al., 1983; Hay et al., 1984). Whether these aggregates are homo-oligomers or associations of precursor with other proteins in the cytosol required for import remains to be established (see section 1.5.3).

Of all the precursors so far studied only the precursor of glutamate dehydrogenase has been reported to have enzymatic activity (Felipo et al., 1983).

Possible roles for the NH_2-terminal extension sequence include holding the protein in a suitable conformation in the cytosol for proper presentation to mitochondria, or to protect it from proteolysis or, in the majority of cases, to ensure that it is enzymically inactive, perhaps by altering the active site or by preventing cofactor binding. Evidence for the last idea comes from the observation that some precursors have failed to bind to affinity columns specific for the mature protein (Kalousek et al., 1984; Ono et al., 1985).

1.3.2 INTRACELLULAR SITE OF SYNTHESIS OF MITOCHONDRIAL PROTEINS

Determining the subcellular site of synthesis of mitochondrial precursors has been central to the question of how mitochondria take up proteins in vivo.

Early studies on the biosynthesis of proteins such as cytochrome c and glutamate dehydrogenase were carried out in vivo by injecting animals with radiolabelled amino acids and following the distribution of radioactive proteins into various subcellular fractions. In general the highest specific activities at early time points were found in the microsomal fraction.

The site of synthesis of glutamate dehydrogenase (GDH) was also studied by isolating free and membrane-bound polysomes from the livers of rats injected with [^{3}H]leucine. Nascent chains released from the ribosomes were screened for nascent glutamate dehydrogenase with pepsin-digested anti-GDH. A preferential reaction of anti-GDH with nascent chains from membrane-associated polysomes was observed. This data suggested that synthesis of mitochondrial precursors occurred on

polysomes associated with microsomal membranes.

However there are now serious reservations regarding the validity of these results. One concerns the selective redistribution of newly-synthesised mitochondrial protein during tissue homogenisation. In fact, it has been shown that radiolabelled protein, added to liver homogenates, becomes preferentially associated with the microsomal membranes in a non-specific fashion. The second concerns the immune assays on nascent chains which tend to form aggregates and could result in non-specific precipitation. Indeed the relationship of the nascent chains to glutamate dehydrogenase was never proven.

An alternative approach has been used by several laboratories to determine the subcellular site of synthesis of these mitochondrial proteins. Free or membrane-bound polysomes or polyadenylated mRNA isolated from these classes of polysomes were translated in cell-free systems and the translation products screened immunochemically for mitochondrial proteins. The translation of cytochrome c, porin and subunit IX of the mitochondrial ATPase complex of N. crassa (Zimmermann et al., 1979a; Freitag et al., 1982b; Schmidt et al., 1983b); the outer membrane protein OMM35 of rat liver and several matrix enzymes of higher eukaryotes (Raymond & Shore, 1979; Morita et al., 1981; Shore et al., 1981; Mihara et al., 1982b; Sonderegger et al., 1982) and of twelve mitochondrial precursors in yeast (Suissa & Schatz, 1982) occurs predominantly on free ribosomes. This suggests, at least for these proteins, a posttranslational mode of import into mitochondria.

Further evidence supporting a posttranslational import mechanism comes from studies in vivo. Fractionation of 'pulse-labelled' cells reveals that appearance of radioactive protein in mitochondria shows a

characteristic lag phase as compared to the kinetics of labelling of cytosolic proteins (Hallermeyer et al., 1977). This suggests that newly-synthesised mitochondrial proteins have to pass through an extra-mitochondrial precursor pool before entering the organelle. This pool has been localised to the cytoplasm and at early stages of the 'pulse', precursor forms are found here whereas mature forms are located inside the organelle (Hallermeyer et al., 1977; Raymond & Shore, 1981; Jaussi et al., 1982; Mihara et al., 1982b; Mori et al., 1981a; Reid & Schatz, 1982). The half-lives of precursor molecules in the cytosol are very short, generally of the order of 1-2 min, although these half-lives are probably not constant since pool sizes vary depending on physiological conditions. Factors which cause a decrease in the rate of protein synthesis will also tend to decrease the size of the precursor pools (Reid & Schatz, 1982).

Observations of cytoplasmic polysomes attached to the mitochondrial outer membrane in cycloheximide-treated yeast cells (Ades & Butow, 1980a) cannot easily be reconciled with free ribosomes being the exclusive site of synthesis of mitochondrial protein. If these polysomes are allowed to complete their nascent chains in an in vitro "read-out" system they apparently release their products into the interior of the mitochondrion (Ades & Butow, 1980a). However, it is not clear whether translocation occurs before completion of the polypeptide chain. Compared to mRNA isolated from free polysomes, mRNA extracted from these mitochondrially-associated polysomes was found to be enriched in mRNAs for mitochondrial proteins (Reid & Schatz, 1982). However, in no case was the total amount of mRNA for a particular mitochondrial protein found to be preferentially, or exclusively,

associated with the polysomes bound to the mitochondria.

It is possible that under conditions which prevent protein synthesis, some nascent chains, with their attached ribosomes may bind to mitochondria, but whether those chains actually span the membrane during translocation has yet to be proven. There is no firm evidence to suggest that these bound polysomes are essential for importing mitochondrial proteins.

While binding of cytoplasmic polysomes to the mitochondrial surface does not seem to be obligatory for the import process, this binding may reflect the specificity of the interactions between precursor polypeptides and mitochondria and as such may serve as probes to help in identifying mitochondrial import receptors. Indeed cytoplasmic ribosomes are preferentially attached to regions of close contact between the inner and outer mitochondrial membranes (Kellems et al., 1975). Such 'contact sites' may be entry sites of nuclear-encoded polypeptides into the organelle.

1.4 INTERACTION OF PRECURSORS WITH BINDING SITES ON MITOCHONDRIA

Following their translation in the cytosol, precursors of mitochondrial proteins must find their way to and into mitochondria. The first step in the transfer process requires the recognition of precursors by the cytoplasmic face of the outer membrane.

1.4.1 PRECURSORS ARE BOUND TO MITOCHONDRIA VIA SPECIFIC RECEPTOR SITES

In order to study the interaction between precursors and mitochondria it is necessary to inhibit translocation. It is also essential to have purified precursor in sufficiently large amounts to monitor its binding to specific mitochondrial recognition sites.

One protein which fulfils these requirements is cytochrome c of N. crassa. Although there is no size difference between the precursor and mature forms, they can be differentiated by non-crossreacting antibodies. The precursor, apocytochrome c, can be prepared in large amounts from holocytochrome c by chemical means (Hennig et al., 1983). It can then be radiolabelled by reductive methylation, bound to mitochondria, translocated into the organelle and converted to holocytochrome c by addition of haem. Translocation can be inhibited by the haem analogue deuterohemin (Hennig & Neupert, 1981). Under these conditions, bound, radioactive apocytochrome c can be exchanged for unlabelled apocytochrome c but not for holocytochrome c. Ligand binding to these sites involves some species specificity. In competition studies equine apocytochrome c was ten-fold less effective in displacing N. crassa apocytochrome c than the homologous protein and apocytochrome c from Paracoccus denitrificans was not effective at all (Hennig et al., 1983). Precursor binds in a saturable manner to high affinity receptors ($K_a = 2.2 \times 10^{-7} M$) which, from Scatchard analysis, are present at 90 pmol/mg mitochondrial protein. This association constant is comparable to the constants determined for polypeptide hormones (Hennig & Neupert, 1983). The observations that bound apocytochrome c is sensitive to proteases and that trypsin treatment of mitochondria prevents binding and import of apocytochrome c

suggests that these receptor molecules are proteins located at the cytoplasmic surface of mitochondria.

Once inhibition of haem attachment is relieved by addition of excess protohaem these bound precursors are translocated and converted to holocytochrome c, implying a functional role for these binding sites. Thus it appears that the mitochondrial binding sites not only selectively interact with apocytochrome c but may also orientate it properly with respect to the membrane to facilitate the initial stages of translocation.

To prove, convincingly, the identity of a particular component as the mitochondrial receptor for apocytochrome c requires its isolation and demonstration of the same high affinity and specificity of binding as intact mitochondria. Preliminary reports state that the protein responsible for high affinity binding of apocytochrome c to N. crassa mitochondria has been purified to homogeneity and exhibits such properties (Harmey & Neupert, 1984; Schmidt & Neupert, 1984; Zwizinski et al., 1984).

1.4.2 MORE THAN ONE TYPE OF RECEPTOR IS PRESENT ON THE MITOCHONDRIAL SURFACE

The search for receptors for mitochondrial proteins other than cytochrome c has been limited by the availability of precursors in only minute amounts. However the existence of such receptors is suggested from the following evidence.

Precursors bound to mitochondria under conditions where translocation is prevented e.g. de-energised mitochondria (Zwizinski et al., 1983), can be processed and internalised once import

conditions are restored. It seems likely, therefore, that this binding is functionally related to import of proteins into mitochondria.

Competition studies have revealed that excess apocytochrome c does not interfere with the binding of various other precursors e.g. ADP/ATP carrier, ATPase subunit IX or cytochrome c_1 (Zimmermann et al., 1981; Teintze et al.,1982), indicating the presence of more than one kind of receptor.

That these receptor molecules are exposed to the cytoplasmic surface is revealed from the evidence that treatment of mitochondria with protease inhibits their ability to bind and import precursors (Gasser et al., 1982a; Argan et al., 1983; Zwizinski et al., 1984) and also from the fact that bound precursors are sensitive to proteases (Freitag et al., 1982b; Riezman et al., 1983b).

Different import mechanisms are also suggested by the different import requirements. Neither cytochrome c, the ADP/ATP carrier or isopropylmalate synthase are synthesised as larger precursors but import of the latter two requires energy. Also cytochrome c and cytochrome c_1, which are both destined for the same compartment, have widely different mechanisms of import. Cytochrome c is synthesised with the same M_r as the mature form and is taken up into the inter-membrane space without a detectable energy requirement. Cytochrome c_1 on the other hand is made as a larger M_r precursor, and undergoes an energy dependent import step involving two proteolytic cleavages as will be discussed later (section 1.6.2).

Attempts have been made to study precursor binding to isolated outer membrane vesicles (Riezman et al., 1984a,b). This binding displays many of the characteristics of a receptor mediated event. It is rapid and reversible, both ligand and membrane specific and is

located on the cytoplasmic surface of the mitochondrial outer membrane. This binding activity can be reconstituted from solubilised membranes but unfortunately saturable binding or limited binding sites cannot be shown owing to the minute amounts of available precursor.

Ono & Tuboi (1985) have reported partial purification of the receptor protein essential for import of pre-ornithine aminotransferase into mitochondria. The purification procedure involved ammonium sulphate fractionation, followed by gel filtration, of solubilised outer membrane of rat liver mitochondria. However the composition of the fraction containing this receptor was not shown.

Import of proteins into the outer membrane is likely to occur by a different mechanism from import into the internal mitochondrial compartments. It is not accompanied by proteolysis of larger M_r precursors, nor does it require protease-sensitive binding sites or an energised inner membrane (Freitag et al., 1982b; Mihara et al., 1982a; Gasser & Schatz, 1983). Still import is membrane specific and appears to be by far the simplest of mechanisms discovered so far.

It seems unlikely that each precursor has its own specific receptor. Indeed the electrophoretic profiles of the outer membrane would not support such a view. The existence of a limited number of different and conserved recognition mechanisms is suggested by the following two observations. Transfer of proteins in vitro does not exhibit species specificity e.g. pre-ornithine carbamyltransferase from rat liver can be imported into mitochondria from pigeon, frog and carp liver (Schleyer et al., 1982; Teintze et al., 1982; Schmidt et al., 1983a; Takiguchi et al., 1983). Also precursors from one cell type can be imported into mitochondria from another cell type which do

not contain this protein *in vivo*, indicating that translocation and processing are common for different mitochondrial proteins (Morita *et al.*, 1982; Mori *et al.*, 1981b). These results suggest that there are possibly common features of the uptake machinery which are neither specific for one species nor specific for individual precursor proteins of the same species.

1.4.3 WHICH PART OF A PRECURSOR IS RECOGNISED BY MITOCHONDRIAL RECEPTORS

Mature proteins do not compete with their precursors for binding to mitochondria, nor are mature, or *in vitro* processed, proteins taken up into the organelle. Although there have been reports of incorporation into mitochondria of mature aspartate aminotransferase (Passarella *et al.*, 1984), this is controversial especially as a precursor form of this protein has been identified (Sonderegger *et al.*, 1982). Some feature of the precursor, therefore, as opposed to the mature form, allows binding of the precursor to mitochondria and its subsequent processing and internalisation.

Mutual recognition between receptors and precursors which do not contain an additional sequence must obviously be via some sequence present in the mature protein. Cytochrome c provides important information in this respect. Since incorporation of cytochrome c is mediated by a mechanism which recognises structural features of the newly-synthesised molecule, these features must be contained in a segment which is masked in the holocytochrome in its native conformation.

Competition studies, employing cyanogen bromide fragments of cytochrome c, have localised the receptor binding 'signal' to a

segment that encompasses, evolutionarily, the most highly conserved region of the protein i.e. residues 70-80 by standard nomenclature (Matsuura et al., 1981). It is interesting in this respect to note that this invariant sequence of eukaryotic cytochromes is not present in cytochrome c of Paracoccus denitrificans which, as has been mentioned already (section 1.4.1), fails to compete with the homoprotein for binding to N. crassa mitochondria.

Recent studies have identified the sequences involved in targetting two outer membrane proteins of yeast to mitochondria: a 70000 M_r protein of unknown function and the pore forming 29000 M_r protein, porin, neither of which are made as larger precursors.

Initial attempts to clone the gene for the 70000 M_r protein resulted in isolation of a truncated gene which had lost a large part of its 3' end during the cloning procedure. The truncated polypeptide, lacking 203 amino acids at the C-terminus, is still found associated with mitochondria, suggesting that regions in the NH_2-terminal 2/3 of the 70000 M_r protein, are responsible for addressing and anchoring the protein to mitochondria (Riezman et al., 1983c). More specific localisation of this addressing and anchoring region has been achieved by deleting various regions throughout the polypeptide chain and also by fusing amino terminal regions of different length to β-galactosidase, introducing these altered genes into yeast and determining the intracellular fate of the polypeptide product. This has revealed that the information is located in an amino terminal region accounting maximally for 7% of the protein. This region contains a typical transmembrane segment, of hydrophobic amino acids, between residues 9-38, flanked by charged amino acids. A role in

membrane anchoring has been assigned to this region, whereas the sequence responsible for targetting proteins to mitochondria is proposed to include the 11 amino-terminal amino acids (Hase et al., 1984). Comparison of the amino acid sequence of this region with the NH_2-terminal region of the outer membrane protein, porin, reveals structural similarities between the two, providing circumstantial evidence that the NH_2-terminal portion of porin, like that of the 70000 M_r protein, functions as both targetting and anchoring signals (Mihara & Sato, 1985).

In the case of proteins synthesised as larger precursors, the presequences are required for mitochondrial import. Thus, with one possible exception (Passarella et al., 1984) mature proteins are not taken up into mitochondria (Riezman et al., 1983b). To test the hypothesis that the presequence contains sufficient information to direct the import of precursors by mitochondria, nucleotide sequences corresponding to leader sequences have been fused to genes for cytosolic proteins and the ability of mitochondria to import these chimeric proteins has been examined.

Hurt et al.(1984a,b) have shown that as little as 23/25 amino acids of the leader sequence of cytochrome c oxidase subunit IV is sufficient to transport cytosolic dihydrofolate reductase into the mitochondrial matrix in vitro. The leader sequence of the human matrix enzyme, ornithine carbamyltransferase, is also sufficient to allow import and processing of cytosolic dihydrofolate reductase both in vitro and in vivo in a CHO-derived cell line (Horwich et al., 1985a). Even β-galactosidase is not restricted in its ability to cross a eukaryotic membrane when fused to the presequence of the nuclear-encoded β-subunit of the mitochondrial ATPase (Schekman, 1985).

A similar situation occurs in chloroplasts, where the fusion of the transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase to a bacterial gene produces a hybrid protein which is taken up and processed in chloroplasts of tobacco plants and into isolated chloroplasts *in vitro* (Van den Broeck *et al.*, 1985; Schrier *et al.*, 1985). These results seem to eliminate the possibility that translocation involves some special features of the precursor polypeptide in addition to the NH_2-terminal targetting sequence. It will be of interest to determine the characteristics of these presequences which are important for recognition: charge, secondary and/or tertiary structure. Recent results infer that the arginine residues of the human ornithine carbamyltransferase leader sequence are related to function (Horwich *et al.*, 1985b). This is not surprising as a basic amino acid composition is a striking feature of all leader sequences reported to date (Hay *et al.*, 1984).

1.5 TRANSLOCATION OF PRECURSORS INTO MITOCHONDRIA

After selective binding of precursors to mitochondria, the next stage involves translocation into one of the four mitochondrial subcompartments. Virtually nothing is known about how translocation is accomplished. An understanding of the precise mechanism would require detailed knowledge concerning the conformation of precursors and the molecular events initiated by specific interaction with the membrane. One intriguing clue to the mechanism of translocation is provided by the observation that import of precursors is an energy-dependent process.

1.5.1 UPTAKE OF MOST PRECURSORS INTO MITOCHONDRIA REQUIRES A MEMBRANE POTENTIAL

Posttranslational transfer of proteins into the mitochondria and their subsequent processing is blocked in whole cells or isolated mitochondria treated with uncouplers of oxidative phosphorylation (Mori et al., 1981b; Reid & Schatz, 1982; Teintze et al., 1982). As already described (section 1.4.2), these uncouplers inhibit transport of precursors into mitochondria but do not affect their binding to the outer membrane. Observations that proteins such as the ADP/ATP carrier and isopropylmalate synthase, which are not synthesised as larger precursors, still require energy for import, suggested that it was the translocation event and not processing which was energy dependent (Zimmermann et al., 1981; Hampsey et al., 1983). This was confirmed by evidence showing that processing of the β-subunit of F_1-ATPase by lysed mitochondria was not dependent on addition of an energy source (Gasser et al., 1982a).

Early work with yeast spheroplasts revealed that import and cleavage of several mitochondrial precursors was blocked by the uncoupler CCCP, or by depleting matrix levels of ATP in non-respiring yeast. From these results it is impossible to distinguish between a requirement for ATP and the need for a membrane potential, since, according to the chemiosmotic hypothesis, a membrane potential can generate ATP from ADP and P_i or ATP hydrolysis can generate a membrane potential. From the observation that uptake and processing still occur in rho^- mutants of yeast, which cannot form a membrane potential because they lack both a functional respiratory chain and a proton-translocating ATPase, the authors concluded that matrix ATP was

probably the immediate energy donor (Nelson & Schatz, 1979).

Definitive evidence to the contrary has been obtained recently using N. crassa, rat liver (Schleyer et al., 1982) and yeast mitochondria (Gasser et al., 1982a). These studies have employed an in vitro import assay in which precursors synthesised in vitro are incubated with isolated mitochondria. Translocation is checked by showing inaccessibility to added protease and correct processing by immune precipitation and SDS-PAGE to compare the M_r value of the processed precursor with the mature form.

In one study, conditions were employed in which the level of matrix ATP was elevated but the membrane potential was dissipated. Under those conditions precursors to subunit IX of ATPase and the ADP/ATP carrier were not imported into mitochondria. On the other hand, conditions were created where the matrix ATP was low but the membrane potential was not destroyed, and in this case protein import into the organelle was inhibited. Matrix levels of ATP in the presence of these various reagents were measured indirectly by following a reaction requiring ATP in the matrix, namely mitochondrial protein synthesis. These results indicate that it is the electrochemical potential and not intramitochondrial levels of ATP which is the primary energy source (Schleyer et al., 1982).

Confirmation of this conclusion was provided by studies on yeast mitochondria (Gasser et al., 1982a). In an in vitro import system, dependent on the addition of ATP, it was shown that import was inhibited by both carboxyatractyloside and oligomycin, inhibitors of ATP uptake and ATP hydrolysis respectively. This indicated that in order for import to occur ATP must be taken up into the matrix of mitochondria and hydrolysed. The most likely explanation for this

would be that ATP is used to generate an electrochemical gradient across the inner mitochondrial membrane. Indeed this import is abolished, if the gradient is collapsed with CCCP or valinomycin, in the presence of oligomycin to prevent the ATPase activity generating a membrane potential.

The electrochemical potential gradient is a sum of two components: a proton gradient and a membrane potential generated from the separation of charged species across the inner membrane. Observations that nigericin, which causes breakdown of the proton gradient without affecting the membrane potential, does not inhibit import and processing (Schleyer et al., 1982) and that valinomycin, which dissipates the membrane potential, does (Schleyer et al., 1982; Gasser et al., 1982a), seem to indicate that it is the membrane potential and not the proton gradient which is necessary to drive uptake of precursors.

An electrochemical potential is also required for the translocation of polypeptides across bacterial membranes (Date et al., 1980). Since the potential has the same polarity in mitochondria as in bacteria whereas the movement of proteins occurs in opposite directions, it is unlikely that the transmembrane potential acts as a simple electrophoretic gradient. It may induce interactions of components of the membrane or alter protein conformation to allow a polypeptide to cross the membrane. Another possibility is that it could labilise the lipid bilayer and facilitate transmembrane movement of polypeptides via transient formation of inverted lipid micelles (Cullis & de Kruijff, 1979). The lipids of the mitochondrial membranes may have a role in translocation but this has not been extensively

investigated as yet.

1.5.2 NOT ALL PROTEINS REQUIRE A MEMBRANE POTENTIAL FOR IMPORT INTO MITOCHONDRIA

Not all proteins require the presence of a membrane potential for import into mitochondria. Proteins which insert into mitochondria in an energy-independent fashion include cytochrome c (Zimmermann et al., 1981), and the proteins of the outer membrane (Gasser & Schatz, 1983; Riezman et al., 1983b). In both cases it could be argued that there is no reason for such a requirement since transport across the inner membrane is not required. However, other inter-membrane space proteins such as cytochrome b_2 and cytochrome c_1 in yeast, are imported only into energised mitochondria (Gasser et al., 1982b). Substantial evidence has been presented to support an import pathway for these proteins which involves a transient interaction with the inner membrane and a two step proteolytic processing mechanism (see section 1.6.2).

Attachment of haem to apocytochrome c is required for completion of import of the precursor into mitochondria. The covalently attached haem strongly affects the conformation of the polypeptide chain and it has been suggested that this conformational change provides the driving force to pull the cytochrome through the outer membrane (Korb & Neupert, 1978).

Haem addition is also required for completion of import and processing of cytochrome c_1 (Gasser et al., 1982b) and δ-aminolevulinate synthase (Ades, 1983) but not for insertion of cytochrome b_2 (Daum et al., 1982). This further illustrates the diversity of mechanisms of translocation existing for mitochondrial

proteins.

Import of proteins into the outer membrane differs in three respects from other mitochondrial proteins: these proteins are not synthesised as larger precursors, with one possible exception in rat liver (Shore et al., 1981); they do not require an electrochemical gradient across the inner membrane for import into mitochondria and their import does not seem to require protease-sensitive sites on the outer membrane (Gasser & Schatz, 1983; Freitag et al., 1982b). Membrane specific insertion has been demonstrated: porin inserts exclusively into mitochondria and is not incorporated into microsomes (Gasser & Schatz, 1983). When isolated mitochondria are incubated with in vitro synthesised precursors at $4^{\circ}C$ a large proportion of the porin is bound to the mitochondria in a protease-sensitive form indicating that binding and integration are separate events. As already mentioned (section 1.3.1), the precursor of porin has a different sensitivity to proteases from the mature form suggesting that there must be a conformational difference between the two. This property of acquired protease resistance can be used to check for integration into the membrane.

Recently the nuclear gene coding for a major 70000 M_r outer membrane protein in yeast has been cloned and sequenced (Hase et al., 1983). From the deduced protein sequence a striking feature of the NH_2-terminal region is a typical transmembrane segment and a strongly basic region which may function as an addressing signal.

The various mechanisms which catalyse the translocation of proteins into the mitochondria seem to depend in part, on the presence or absence of an NH_2-terminal extension sequence, on the final

destination of the protein in the mitochondria and probably on characteristics of the polypeptides themselves.

1.5.3 CYTOSOLIC FACTORS INVOLVED IN TRANSLOCATION

Several recent reports have centred on the requirement for cytosolic factors in the import of precursors into the mitochondria.

In a study concerning requirements for *in vitro* uptake of ornithine carbamyltransferase, synthesised in a rabbit reticulocyte lysate cell free system, into rat heart mitochondria, Argan *et al.* (1983) observed that import was inhibited if the lysate was subjected to gel filtration on Sephadex G25 prior to incubation with mitochondria. Confirmation of this was reported by Ohta & Schatz (1984) who observed a similar effect on import of cytochrome b_2 into yeast mitochondria. Addition of non-radioactive reticulocyte lysate to the system stimulated import of the *in vitro* synthesised cytochrome. It was concluded that the factor in the lysate responsible for this behaviour was either very small or reacted in a non-specific manner (ionically or hydrophobically) with the gel matrix (Argan *et al.*, 1983).

Further evidence as to the nature of the component involved was achieved in a study on *in vitro* import of ornithine carbamyltransferase into rat liver mitochondria. The stimulatory activity was retained in a dialysed reticulocyte lysate supernatant fraction but was lost on trypsin treatment or heat treatment at 100°C for 2 min (Miura *et al.*, 1983). It was also reported that, in the absence of this factor, *in vitro* synthesised precursor was found in the supernatant fraction, not associated with mitochondria,

suggesting the factor is responsible for binding or transport and not processing.

An alternative approach involved purification of the precursor to the β-subunit of the F_1 ATPase from the rho^- yeast mutant grown in the presence of CCCP (Ohta & Schatz, 1984). This purified precursor was processed very inefficiently by isolated yeast mitochondria although the reaction could be stimulated by reticulocyte lysate or a cytosolic fraction of yeast. As the stimulatory agent can be precipitated by ammonium sulphate and inactivated by trypsin, it implies the involvement of a protein. Gel filtration revealed that the stimulatory activity migrated with an apparent M_r of 40000. This factor is unlikely to function in a manner analogous to the SRP (section 1.2.1) as this complex prevents completion of polypeptide chains in the cytosol in the absence of target membranes and mitochondrial proteins are usually transported posttranslationally. One hypothesis would be that this cytosolic factor binds to precursors and triggers their correct association with the appropriate receptors. Until the agent responsible has been purified to homogeneity, its exact nature, mechanism of action and biological function will remain unknown.

1.6 PROCESSING OF PRECURSORS TO THEIR MATURE FORMS

During or shortly after translocation, precursors carrying NH_2-terminal extension sequences are proteolytically cleaved to their mature forms by a neutral, chelator-sensitive, matrix protease. This enzyme has been found in mitochondria from yeast (McAda & Douglas, 1982; Bohni *et al.*, 1983); rat liver (Mori *et al.*, 1980) and *N. crassa* (Schmidt & Neupert, 1984).

Characteristics of the yeast enzyme include a pH optimum of 7.5, inhibition by chelating agents such as EDTA, GTP and 1,10-phenanthroline, which is reversible on readdition of Zn^{2+} or Co^{2+}, and insensitivity to serine protease inhibitors and sulphydryl reagents (Bohni et al., 1983). The protease is specific for mitochondrial precursors and does not cleave either non-mitochondrial proteins or denatured precursors. This suggests, that some particular conformation of the precursor, is required for correct cleavage. However, the protease processes a variety of in vitro synthesised mitochondrial precursors and demonstrates little species specificity. Correct processing of subunit V of cytochrome c oxidase by this enzyme has been demonstrated by comparing the amino acid sequence of the NH_2-terminus of mature and in vitro synthesised precursor treated with partially-purified protease (Cerletti et al., 1983). Further purification revealed an apparent M_r of 115000 by gel filtration. Although this fraction still consists of several protein bands, McAda & Douglas (1982) have reported that the processing activity co-purifies best with the presence of one major 59000 M_r band.

The processing activity in rat liver differs slightly in its sensitivity to inhibitors e.g. it is inhibited by 2mM leupeptin and 2mM antipain, unlike the yeast enzyme. Co-fractionation of this enzyme with markers of the inter-membrane space has been observed but this may reflect cross-contamination of the sub-fractions (Mori et al., 1980).

1.6.1 TWO STEP PROCESSING

Several mitochondrial precursors studied so far are reported to be cleaved to their mature forms in two discrete steps. Cytochrome b_2 in yeast, a soluble component of the inter-membrane space, is synthesised initially as a larger M_r precursor of 68000, which is taken up into the mitochondria in an energy dependent fashion and cleaved to an intermediate form of M_r 63000. This intermediate form, observed when *in vitro* synthesised cytochrome b_2 is incubated with isolated mitochondria or partially-purified protease and also during *in vivo* 'pulse-chase' studies in yeast spheroplasts (Reid *et al.*, 1982; Daum *et al.*, 1982), is thought to be bound to the outer surface of the inner membrane (Daum *et al.*, 1982). Conversion of the intermediate to the mature 58000 M_r form of cytochrome b_2 is brought about by a protease, distinct from the matrix protease, which is insensitive to 1,10-phenanthroline and probably associated with the outer aspect of the inner mitochondrial membrane (Daum *et al.*, 1982).

Processing of cytochrome c_1 is similar in many respects to that of cytochrome b_2 except that the second proteolytic cleavage requires energy (Teintze *et al.*, 1982) and is dependent upon the addition of haem to the intermediate form (Gasser *et al.*, 1982b; Ohashi *et al.*, 1982). Presumably this covalent attachment of the haem brings about a conformational change which renders the protein sensitive to the second protease. Following this second proteolytic cleavage, unlike cytochrome b_2, cytochrome c_1 remains attached to the inner mitochondrial membrane.

Although no intermediate form of cytochrome c peroxidase has been observed *in vivo* its conversion is thought to take place via

a two step mechanism. It is synthesised as a larger precursor (Maccecchini et al., 1979b), and cleaved to an intermediate form by the partially-purified matrix protease (Gasser et al., 1982b). This processing in vivo is blocked by 1,10-phenanthroline (Reid et al., 1982) again suggesting a role for the matrix protease. Maturation of cytochrome c peroxidase is much slower than that of cytochrome b_2 in vivo. After a 3 min 'pulse' the cytochrome c peroxidase precursor appears to be located inside the mitochondria, bound to the inner membrane, since a subsequent chase is insensitive to uncouplers of oxidative phosphorylation (Reid et al., 1982). Failure to observe an intermediate form could be explained if the first cleavage step was rate limiting, as the intermediate would then not be detected for kinetic reasons.

A recent report revealed that subunit IX of the N. crassa ATPase was processed in two steps, both of which were assumed to be catalysed by the same enzyme (Schmidt & Neupert, 1984).

An apparent intermediate form of ornithine carbamyltransferase is observed upon import and processing by isolated mitochondria (Mori et al., 1980; Morita et al., 1982; Argan et al., 1983). This intermediate form is not observed in vivo in isolated hepatocytes (Mori et al., 1981a), does not chase into isolated mitochondria and is located outside the mitochondria (Argan et al., 1983). A protease has been partially purified from rat liver mitochondria which cleaves the precursor of ornithine carbamyltransferase only to the intermediate form (Mori et al., 1980). The role of this protease and that of the 37000 M_r polypeptide intermediate in processing and transport remains to be determined.

Other types of posttranslational modification, involved in the

conversion of precursor to mature forms, include covalent attachment of haem or flavin nucleotides, methylation of amino-acyl side chains and acetylation of the mature NH_2-terminus. In general, although these modifications are essential for protein function, the role played by the enzymes responsible for cofactor attachment in precursor import is unclear.

1.7 ASSEMBLY OF NEWLY-SYNTHESISED PROTEINS INTO FUNCTIONAL UNITS

The final stage in the import process, following translocation and processing, is assembly of newly-synthesised polypeptides into functional units inside the mitochondrion. This may involve association to form homooligomers or heterooligomers or, in the case of several respiratory complexes of the inner membrane, the association of cytoplasmically-synthesised polypeptides with mitochondrially -synthesised polypeptides.

In comparison to the other steps in the pathway, there have been relatively few reports on assembly of mitochondrial proteins. One reason for this is probably the difficulty involved in determining suitable criteria to define correct assembly.

A few studies have addressed the problem of correct intra-mitochondrial location of newly-synthesised proteins which could be considered the first step in the assembly process. Several precursors of matrix, inter-membrane space and inner membrane proteins have been shown to be taken up by isolated yeast mitochondria into their correct locations (Gasser et al., 1982a) and newly-synthesised carbamylphosphate synthetase is imported into the matrix fraction of

rat liver mitochondria (Campbell et al., 1982).

Correct insertion into the appropriate membrane has been inferred for the outer membrane, porin, and the inner membrane, ADP/ATP carrier, by virtue of the different structural properties of the precursor and mature forms as mentioned in section 1.3.1 (Zimmermann & Neupert, 1980a; Gasser & Schatz, 1983; Freitag et al., 1982b). An increase in specific activity of carbamylphosphate synthetase in isolated mitochondria incubated with pre-carbamylphosphate synthetase, which is enzymatically inactive, reveals an assembly of the mature polypeptide into the active dimeric form (Campbell et al., 1982). Evidence has also been presented that translocated and processed ornithine carbamyltransferase can form trimers resembling the native enzyme which bind to a substrate analogue affinity column (Kalousek et al., 1984). Neither the precursor nor the reported intermediate of ornithine carbamyltransferase bind to this column. In a study on the assembly of F_1-ATPase in isolated yeast mitochondria, Lewin & Norman (1983) showed that newly-synthesised α, β & δ-subunits at least, appear to combine to form heterooligomers. It has also been observed that antibody to F_1-ATPase can immunoprecipitate subunit IX, an integral membrane subunit of the ATPase, which suggests an association of subunit IX with the F_1 section of the mitochondrial ATPase (Schmidt et al., 1983b).

Assembly of some of the respiratory complexes of the inner membrane and the mitochondrial ribosomes requires products of both cytoplasmic and mitochondrial protein synthesis. Little is known about the mechanism or control of assembly of these complexes. Specific inhibition of either cytoplasmic or mitochondrial protein synthesis should ultimately result in shortage of part of the components of

these complexes and, consequently, in impaired assembly. The processes co-ordinating the expression of the different genetic systems are largely unknown but there is not tight coupling between the two (de Vries & van't Sant, 1983).

1.8 FUTURE AIMS IN MITOCHONDRIAL BIOGENESIS

It seems likely that future work in this field will rely heavily on techniques of DNA manipulation with the aims of further dissection of sequences reponsible for targetting proteins to the mitochondria and production of large amounts of pure precursor. The latter achievement would allow binding to receptor molecules to be examined in more detail and perhaps a more specific description of the conformation of precursors as opposed to mature proteins to be produced.

Another technique which is likely to feature strongly is genetic manipulation. Yeast strains defective in import and maturation have been produced (Yaffe & Schatz, 1984). Examination of the lesions involved could lead to the identification of components of the import pathway, such as the receptor molecules, proteases responsible for maturation or factors in the cytosol required for import.

Although yeast is the best system in which to carry out the work described above due to the well established nature of yeast genetics, problems which may still be addressed in other systems, such as mammalian cells, include addition of cofactors and assembly of multiprotein complexes.

The ultimate aim of these studies, i.e. the reconstitution of an import and maturation system from purified components, must

surely be realised within the next decade.

1.9 MULTIENZYME COMPLEXES

As stated in section 1.1, eukaryotic cells exhibit a high degree of organisational complexity, being subdivided into a number of organelles, each of which has a unique function and protein composition. The separate functions of each subcellular compartment are interdependent. Specific interaction of cellular components in space and time is important to biological function as is illustrated in the preceeding sections. Probably the best example of this, already cited, is the cotranslational transport of proteins across the endoplasmic reticulum (section 1.2.1): protein synthesis is initiated on free ribosomes (assemblies of protein and RNA) in the cytoplasm; up to a certain time after initiation of protein synthesis, an SRP (again an association of protein and RNA) can bind to the growing chain and prevent further elongation; this SRP-polysome complex then binds to specific proteins in the endoplasmic reticulum resulting in relief of arrest and allowing translation coupled to translocation to proceed. These interactions ensure that the nascent protein appears in the correct cellular location. Basic cellular processes from DNA synthesis to ATP generation depend upon the interaction of proteins.

Metabolic pathways in the cell consist of a number of consecutive reactions, catalysed by individual enzymes functioning sequentially. Thus the product of one reaction is a substrate for the next enzyme in the chain. It has become apparent that these interrelated enzymes are not randomly distributed but are often specifically organised into functional assemblies. Functionally related enzymes may aggregate to

form multienzyme complexes. Physical interactions of the enzymes in these complexes result in a distinctive morphology. The most highly organised multienzyme complexes in the cell are probably the arrays of membrane-bound enzymes of oxidative phosphorylation in mitochondria.

The most commonly studied multienzyme complexes such as the fatty acid synthetase (Lynen, 1972), 2-oxo acid dehydrogenase complexes (Reed, 1974; Perham, 1975) and the multienzyme complexes involved in aromatic amino acid biosynthesis (Yanofsky & Crawford, 1972; Lumsden & Coggins, 1977), are composed of 2-6 enzymes and have M_r values ranging from thousands to millions. All of the associated proteins play an active role and there are no nucleic acids or lipids integrated into the complexes.

Structures of a number of these complexes have been described in great detail while in some cases the individual components have been resolved and reconstituted to form particles resembling the native complex.

Association of proteins to form multienzyme complexes provides opportunities for increased specificity, efficiency and modes of control not possible with structurally-independent enzymes.

In a few cases the activity of one component of a complex is modified by interaction with others. One example of this is the tryptophan synthetase complex of E. coli (Yanofsky & Crawford, 1972), a tetrameric enzyme with subunit structure $\alpha_2\beta_2$. Tryptophan synthetase catalyses the last reaction in the tryptophan pathway:-

Indole-3-glycerolphosphate + L-serine ---→

L-tryptophan + D-glyceraldehyde-3-phosphate 1

The enzyme is also capable of catalysing the two half-reactions:-

Indole-3-glycerolphosphate ---→

Indole + D-glyceraldehyde-3-phosphate 2

Indole + L-serine ---→ L-trytophan 3

Resolution of the complex results in α and β_2 species both of which are catalytically active, α in reaction 2 and β_2 in reaction 3. However, neither are active in the overall reaction. In the assembled complex the overall reaction can take place, the catalytic efficiencies of α and β_2 are much higher and potentially competitive side reactions such as the serine deaminase activity of β_2 are suppressed.

Where intermediates are retained on the surface of the complex this may be regarded as a form of segregation and may exclude competition from enzymes of another pathway. The arom complex of N. crassa provides the best documented example of this kind. N. crassa is capable of synthesising an inducible dehydroquinase, which probably serves a catabolic function, in addition to the constitutive dehydroquinase which is part of the complex (Giles et al., 1967). It has been suggested that because the constitutive enzyme is a component of a multienzyme complex it can compete effectively for the dehydroquinate substrate channelling it preferentially along the biosynthetic pathway. Other complexes for which a channelling function seems plausible, where intermediates are strongly bound to the complex, include the 2-oxo acid dehydrogenase complexes and the yeast fatty acid synthetase.

Association of enzymes within cells may be far more extensive than was originally imagined. Some evidence has been presented which indicates that glycolytic enzymes form a functional particle (Gorringe & Moses, 1978). Also it has been suggested that certain enzymes of the citric acid cycle are physically associated in the cell (D'Souza & Srere, 1983; Sumegi & Srere 1984).

Multienzyme complexes participate in reactions involving a number of different substrates and have been found in a variety of biological systems. The natural occurrence of such complexes is probably fairly frequent. Whether they can be isolated depends on their structural stability under the conditions employed.

Purified complexes provide the opportunity for study of assembly and subunit interactions. Such studies will result in a deeper insight into mechanisms whereby proteins influence the behaviour of other proteins and the integration of protein function. This knowledge can be applied to the principles governing cellular organisation.

1.10 2-OXO ACID DEHYDROGENASE COMPLEXES

Multienzyme complexes which catalyse the lipoic acid-mediated oxidative decarboxylation of 2-oxo acids have been isolated from microbial and eukaryotic cells as functional units of high M_r. Three categories of 2-oxo acid dehydrogenase have been identified: one specific for pyruvate, one specific for 2-oxoglutarate and one responsible for oxidation of branched chain 2-oxo acids. All 2-oxo acid complexes purified to date consist of three catalytic components: a substrate specific dehydrogenase, a lipoate acyltransferase and with one possible exception in the case of Pseudomonas putida (Sokatch

TABLE 1.2 Specific reactions catalysed by the 2-oxo acid dehydrogenase complexes.

COMPLEX	R	SUBSTRATE	PRODUCT
2-oxoglutarate dehydrogenase	$CO_2.CH_2.CH_2-$	2-oxoglutarate	succinyl-CoA
pyruvate dehydrogenase	CH_3-	pyruvate	acetyl-CoA
branched-chain 2-oxo acid dehydrogenase	$(CH_3)_2.CH.CH_2-$	2-oxoisocaproate	isovaleryl-CoA
	$(CH_3.CH_2).(CH_3).CH-$	2-oxo-3-methyl-valerate	2-methyl-butyryl-CoA
	$(CH_3)_2.CH-$	2-oxo-iso-valerate	isobutyryl-CoA

et al., 1981a) a flavoprotein common to all three complexes. These component enzymes self assemble to form ordered, symmetrical structures of distinct morphology. Five coenzymes and prosthetic groups are involved in the sequence of reactions catalysed by the complex: thiamine pyrophosphate, lipoic acid, CoA, FAD and NAD^+.

The overall oxidative decarboxylation reaction can be represented as:-

$$R.CO.CO_2H + CoA + NAD^+ \quad \longrightarrow$$

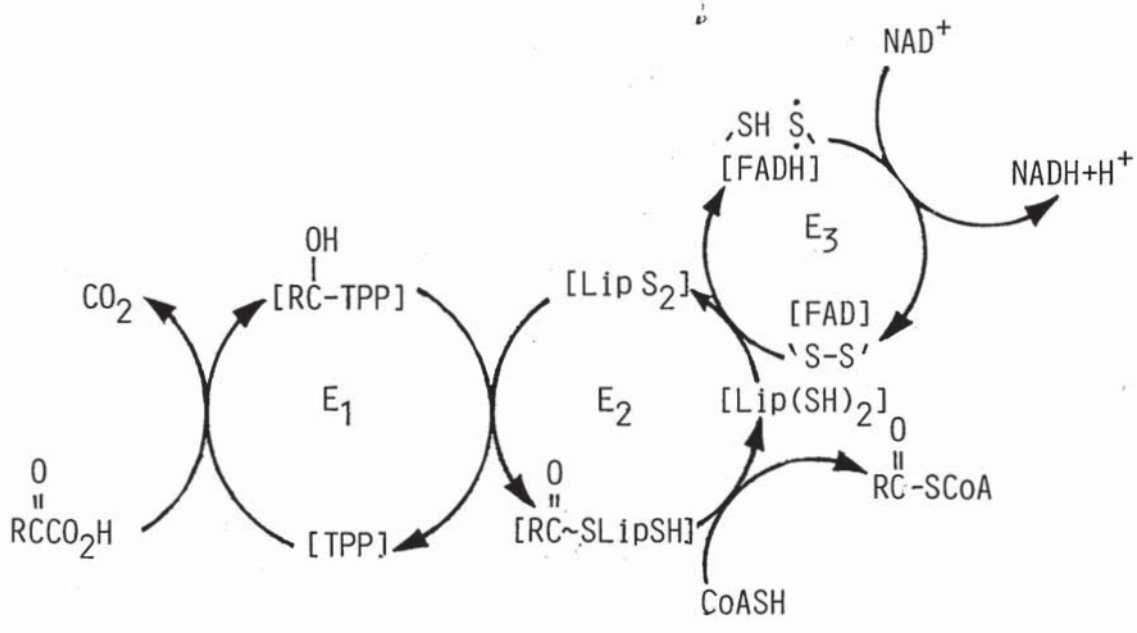
$$R.CO.CoA + CO_2 + NADH + H^+$$

The specific reactions catalysed by the 2-oxoglutarate dehydrogenase complex (OGDC), the pyruvate dehydrogenase complex (PDC) and the branched chain dehydrogenase complex (BCDC) are shown in Table 1.2.

Succinyl-CoA, produced by OGDC, is required for the oxidation of ketone bodies and the biosynthesis of porphyrins, leucine and methionine. PDC provides acetyl-CoA, a key precursor in the biosynthesis of many different lipids, leucine, lysine (in fungi), acetylcholine and a metabolite of the glyoxylate cycle in plants. Oxidation of acetyl-CoA via the citric acid cycle is a major source of reducing power as NADH and energy in the form of ATP. BCDC is involved in the degradation of the branched chain amino acids valine, leucine and isoleucine. In humans, a deficiency of this activity causes Maple Syrup Urine Disease, a major symptom of which is the presence in the urine of high levels of branched chain 2-oxo acids (Chuang et al., 1981).

Purification of each of the 2-oxo acid dehydrogenase complexes has been achieved from several sources. The following lists provide

FIGURE 1.3 Reaction mechanism of the 2-oxo acid dehydrogenase complexes



TPP : thiamine pyrophosphate
Lip : lipoic acid
R : see table 1.2

examples of these. PDC and OGDC have been isolated from E. coli (Koike et al., 1960; Reed & Mukherjee, 1969; Danson et al., 1979), Azotobacter vinelandii (Bosma et al., 1982), bovine heart (Stanley & Perham, 1980), bovine kidney (Linn et al., 1972; Kresze & Steber, 1979), porcine heart (Massey et al., 1960; Koike & Koike, 1976) and Saccharomyces cerevisiae (Hirabayashi & Hirada, 1971; Kresze & Ronft, 1981a,b). In addition, PDC has also been purified from Pseudomonas aeruginosa (Jeyaseelan et al., 1980), Bacillus stearothermophilus (Henderson & Perham, 1980), Bacillus subtilis (Visser et al.,1980; Hodgson et al., 1983) and porcine liver (Roche & Cate, 1977). BCDC has been isolated from Pseudomonas putida (Sokatch et al., 1981b), bovine liver (Danner et al., 1979) and bovine kidney (Pettit et al., 1978).

1.11 STRUCTURE AND FUNCTION OF THE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

The multienzyme system responsible for the oxidation of 2-oxoglutarate has been isolated from the sources listed above as a functional unit with M_r 2.5-2.7 x 10^6. OGDC is composed of multiple copies of three different enzymic activities: 2-oxoglutarate dehydrogenase (EC 1.2.4.2), lipoate succinyltransferase (EC 2.3.1.6) and lipoamide dehydrogenase (EC 1.8.1.4). These, and the corresponding activities from the other 2-oxo acid dehydrogenase complexes, are generally referred to as E1, E2 and E3 respectively. Acting in sequence, these enzymes catalyse the reaction shown in Fig.1.3 (Reed, 1974).

In a reaction involving the coenzyme TPP, E1 catalyses the decarboxylation of 2-oxoglutarate. This enzyme is also responsible for the subsequent reductive acylation of the lipoyl moiety, covalently

bound to E2. Acylation of lipoic acid results in formation of a thioester and opening of the dithiolane ring, generating a free thiol. E2 catalyses the transfer of the acyl group from the lipoic acid to CoA, releasing one of the final reaction products, succinyl-CoA. Reoxidation of the dihydrolipoyl moiety is catalysed by E3. This enzyme contains a covalently bound FAD prosthetic group and a disulphide bridge which are both involved in the reaction mechanism. A chief feature of this reaction is a catalytic intermediate where two electrons are shared between the FAD and the reactive disulphide (Massey, 1960; Massey & Veeger, 1961; Massey, 1963). NAD^+ is the ultimate electron acceptor.

SDS-PAGE reveals that purified OGDC contains three different polypeptide chains. Apparent M_r values for these chains in porcine heart OGDC are 113000, 55000 and 48000 (Koike et al., 1974) and in E. coli OGDC 94000, 54000 and 47000 (Perham & Thomas, 1971; Pettit et al., 1973). Resolution of OGDC into its component enzymes has been achieved by gel filtration in the presence of chaotropic agents (Tanaka et al., 1972; Pettit et al., 1973). This has allowed assignment of the 113000, 55000 and 48000 M_r polypeptides of porcine heart to the functions of E1, E3 and E2 respectively (Koike et al., 1974). Similarly the 94000, 54000 and 47000 M_r proteins of E. coli OGDC were found to correspond to the E1, E3 and E2 activities respectively (Pettit et al., 1973).

Sedimentation equilibrium analysis of the component enzymes revealed that, at normal ionic strength and pH, E1 and E3 exist as dimers whereas the E2 component is isolated as an assembly of high M_r, approx. 1×10^6 (Tanaka et al., 1972; Pettit et al., 1973).

Of the three protein bound cofactors, all of the TPP is found

associated with the E1 component whereas the lipoic acid and FAD were isolated with E2 and E3 respectively.

The three isolated enzymes reassemble spontaneously to produce a large unit resembling the native complex in composition and enzymic activity. Native OGDC has a sedimentation coefficient of approx. 37S (Hirashima et al., 1967; Stanley & Perham, 1980), an M_r of approx. 2.7 X 10^6 (Pettit et al., 1973; Koike & Koike, 1976) and a diameter of 25-26 nm (Ishikawa et al., 1966; Tanaka et al., 1972). Binding studies reveal that E1 and E3 do not interact with each other but each of these enzymes does associate with E2 (Tanaka et al., 1972; Pettit et al., 1973). This suggests that E2 plays a key role in the structural organisation of the complex as well as in its enzymatic activity. From these studies the idea of structural organisation suggested was one of an organised mosaic of enzymes in which each subunit is uniquely located to permit efficient implementation of the consecutive reactions.

Results of electron microscopic studies, X-ray crystallographic studies and sedimentation equilibrium M_r determinations revealed that the E2 core enzyme of OGDC consists of 24, apparently identical, polypeptide chains, grouped into 8 trimeric morphological units, arranged with 432 molecular symmetry in a cube-like particle (DeRosier et al., 1971; Tanaka et al., 1972). Limited proteolysis of E. coli lipoate succinyltransferase revealed that each chain consists of two principal domains (Perham & Roberts, 1981):

1. A compact domain (M_r 36000) which contains the intersubunit binding regions and the transsuccinylase catalytic site. This domain which forms a closed structure, the E2 inner core, determines the stoichiometry and quaternary structure of the

complex;

and

2. A (M_r 11000) fragment bearing the lipoyl groups.

These domains are apparently connected by a hinge region.

The mobility of the flexible 'lipoyl-bearing' domain is revealed by proton NMR spectroscopy where unexpectedly sharp resonance lines are observed superimposed on the broad resonances expected of such a large protein complex (Perham & Roberts, 1981). By analogy with E. coli PDC (Perham et al., 1981), these sharp resonances have been attributed to the high mobility of the lipoyl domains. Indeed all the evidence is consistent with a random coil-like structure for these highly mobile regions of polypeptide chain, though small parts of these regions could have defined three dimensional structure. The existence of part of the lipoyl domain other than the lipoyl-lysine bearing segment is shown by analysis of the structure remaining after trypsin releases the 11000 M_r lipoyl-bearing fragment. A flexible region still remains attached to the inner core (Perham & Roberts, 1981). One possibility is that E2 consists of three domains: the inner core, a structured lipoyl-containing domain and a highly flexible region of random coil which links the two. This structure seems to be present in transuccinylases from bovine heart and bovine kidney also (Kresze et al., 1981; Warwrzynczak et al., 1981), although protease treatment of bovine kidney OGDC, while producing an E2 fragment which remains assembled in a structure of high M_r, apparently damages the E1 and E3 binding sites on E2 resulting in dissociation of these subunits.

From the determination of cofactor content of the purified complex and consideration of the subunit M_r, the stoichiometry of polypeptide chains in the E. coli OGDC was estimated at 10 E1: 24 E2: 10 E3 (Pettit et al., 1973), although E3 contents of 12 chains per 24 E2 subunits were observed on occasions. It was suggested from this that the native ratio is probably E1:E2:E3 = 12:24:12. Titration of OGDC with the transition state analogue inhibitor of E1, thiamine thiazolone pyrophosphate, also indicated the presence of 12 E1 active sites per complex (Angelides & Hammes, 1979). By use of the radio-amidination procedure of Bates et al. (1975), the chain ratio of porcine heart OGDC was estimated as 12 E1: 24 E2: 12 E3 (Koike & Koike, 1982). The possibility cannot be ruled out, however, that OGDC, like E. coli PDC, is heterodisperse. Gilbert & Gilbert (1980) showed that a symmetrical Schleiren peak is not neccessarily unequivocal proof of homogeneity.

Determination of coenzyme content of native OGDC has shown that the porcine heart complex contains 6 moles TPP, 8 moles lipoic acid and 12 moles FAD per mole of complex. This means that every E1 dimer has an associated TPP, every 3 E2 chains contain 1 lipoic acid and each E3 chain contains an FAD group (Tanaka et al., 1972). In the case of the E. coli OGDC the lipoic acid content was determined by measuring incorporation of [4-^{14}C]succinyl groups into E2, following incubation with 2-[5-^{14}C]oxoglutarate or incorporation of N-ethyl[2,3-^{14}]maleimide into the free thiol, generated by reductive acylation of lipoic acid. Each E2 chain was shown to contain one lipoic acid (Collins & Reed, 1977; Angelides & Hammes, 1979). This was confirmed by isotopic dilution analysis of the lipoic acid content performed by White et al. (1980). Accurate determination of the lipoyl

content of transacylases has proved difficult. Variable results have been obtained from microbial assays (used to determine lipoic acid content of porcine heart OGDC) and measurement of content of radioactive lipoic acid in complexes isolated from E. coli grown in the presence of [$^{35}S_2$]lipoic acid (Collins & Reed, 1977).

A key functional role in the co-ordinated sequence of reactions is played by E2. The lipoyl group, covalently bound to this polypeptide, undergoes a series of transformations in the catalytic cycle: reductive acylation, acyl transfer and electron transfer. These involve interaction with prosthetic groups or coenzymes of 3 distinct enzymes and no reaction intermediates are released from the complex. Highly favourable positioning of the active sites of the 3 enzymes must be assumed in order to account for the occurrence of the overall reaction. Lipoic acid was found to be attached via an amide linkage to a lysine residue of E2 (Nawa et al., 1960). Subsequently it was proposed that this provided a flexible arm 1.4 nm long which permitted the lipoyl moiety to rotate between the catalytic sites of all 3 enzymes. Indeed e.s.r. spectroscopy of E. coli PDC, a complex with similar structural and functional properties to OGDC (Reed & Oliver, 1968), indicated the flexibility of these lipoyl moieties (Ambrose & Perham, 1976).

Fluorescence energy transfer measurements of the distance between the catalytic sites of the enzyme components of E. coli OGDC were consistent with a mechanism involving rotation of a single lipoyl group between catalytic sites during the reaction cycle (Angelides & Hammes, 1979). Discovery of flexible lipoyl domains however, has led to a proposal that movement of these domains and not merely the

lipoyl-lysine provides the means to span the physical gaps between the catalytic sites (Stepp et al., 1981).

In titrations of E2-E3 subcomplexes with E1, the overall activity was a linear function of E1 added and maximal activity was observed with chain ratio 12 E1: 24 E2 (Reed & Oliver, 1982). Similar results were observed when OGDC was inactivated by titration of E1 with the transition state analogue, thiamine thiazolone pyrophosphate (Angelides & Hammes, 1979). This indicates that the reaction catalysed by E1 is rate-limiting in the reaction sequence, (Fig. 3).

However, when 9 out of 12 E1 polypeptides of OGDC were inhibited with thiamine thiazolone pyrophosphate, rapid incorporation of approx. 20 succinyl groups per molecule of complex was observed (Collins & Reed, 1977). Treatment of OGDC with dimaleimide in the presence of substrate resulted in cross-linked dimers of E2. Dimaleimide cross-links E2 via succinyldihydrolipoyl or dihydrolipoyl groups. The presence of cross-linked E2 dimers indicates that E2 contains only one lipoyl per chain and that these lipoyl groups can interact (Collins & Reed, 1977). In addition, quantitative removal of lipoyl groups by lipoamidase or lipoyl domains by trypsin and monitoring of residual enzyme activity points to interaction of lipoyl groups. On removal of 50% of lipoyl moieties OGDC retained 75% of its original activity (Stepp et al., 1981). These results suggest each E1 subunit may transfer succinyl groups to more than one lipoyl domain. Computer analysis has suggested a model whereby each E1 services 2 lipoyl groups (Hackert et al., 1983). Although the capacity for succinyl transfer between lipoyl groups is present in the complex, it has not been demonstrated that such transfer is on the main catalytic pathway (Angelides & Hammes, 1979).

Consideration of the structure of OGDC reveals that not all E3 polypeptide chains are positioned identically with respect to the lipoyl domains of E2. Titration of E1-E2 subcomplex with E3 indicates the presence of multiple interconnected pathways from E1 to E3 (Pettit et al., 1973). Therefore it is unlikely that all dihydrolipoyl moieties are re-oxidised by direct interaction with E3 and electron transfer between oxidised and reduced lipoyl groups appears an essential property of the complex (Reed & Oliver, 1982). The geometry of possible arrangements of E3 dimers shows that probably only one thiol disulphide exchange is required to transfer one electron pair to E3.

This active site coupling seems to be a common property of all OGDC and PDC complexes studied so far. Active site coupling obviously has advantages in that under physiological conditions where CoA and 2-oxo acid levels are so low that the joint probability of binding to adjacent sites is very low the reaction can still proceed. In addition it is also neccessary in situations where active sites are not present in equal numbers.

1.12 REGULATION OF 2-OXOGLUTARATE DEHYDROGENASE

Under most metabolic conditions, the reaction catalysed by OGDC is the primary site of control for the segment of the citric acid cycle from 2-oxoglutarate to malate. 2-oxoglutarate is a branch point metabolite of the citric acid cycle which may be oxidised via the citric acid cycle to produce energy or withdrawn from the cycle for energy-requiring biosynthetic pathways. Regulation at this step would ensure that the requirements of the cell for energy in addition to

biosynthetic intermediates are kept in balance.

A general feature of the regulation of OGDC is feedback inhibition by the reaction products succinyl-CoA and NADH which is reversed by CoA and NAD^+ respectively (Garland, 1964; Hansen & Henning, 1966; Parker & Weitzman, 1973). The site of action of NADH is proposed to be located on the E3 subunit (Hansen & Henning, 1966; Parker & Weitzman, 1973), although there may be an additional site for NADH action on the E1 component of OGDC from Acinetobacter lwoffi (Parker & Weitzman, 1973). Lipoate succinyltransferase contains the site for succinyl-CoA inhibition (Koike & Koike, 1976). In addition to acting as a substrate for Acetobacter xylinum OGDC, NAD^+ also appears to act as an allosteric modifier decreasing the Km for 2-oxoglutarate (Kornfeld et al., 1977). In this system positive cooperativity with respect to 2-oxoglutarate has also been observed.

The activity of OGDC is also affected by the presence of adenine nucleotides. ATP has an inhibitory effect on OGDC (Hansford, 1972; Ohne, 1975; McCormack & Denton, 1979; Williamson & Cooper, 1980), whereas AMP or ADP stimulate OGDC activity in all cases studied except E. coli (Wedding & Black, 1971; Parker & Weitzman, 1973; Kornfeld et al., 1977; McCormack & Denton, 1979; McCormack & Denton,1981). This effect on enzymatic activity is achieved by an alteration in the Km for 2-oxoglutarate. ATP does not act by causing phosphorylation of OGDC as is true in the cases of PDC and BCDC (Reed & Yeaman, 1985). The effect appears to be mediated by adenine nucleotides binding to a site on E1 (Kornfeld et al., 1977). The specificity of this binding is demonstrated by the preference for adenine nucleotides, the presence of 2' and 3' -OH groups and the presence of a phosphate group attached

to the cyclo-furan ring (Wedding & Black, 1971). AMP is an indicator of a low energy state in the cell, its concentration varying inversely with that of ATP. Control of OGDC by adenine nucleotides may serve as a physiological mechanism for regulating energy metabolism by increasing flux around the citric acid cycle and away from biosynthetic pathways when the energy demands of the cell require it.

Inhibitory effects of succinyl-CoA and NADH may provide additional mechanisms whereby flux could be related to the oxidation-reduction state of the cell and via succinyl-CoA synthetase to the phosphorylation state of cellular nucleotides.

The activity of porcine heart OGDC has been shown to be inhibited by branched chain 2-oxo acids suggesting that the complex is regulated by relative levels of various 2-oxo acids, including branched chain 2-oxo acids derived from intermediary metabolism of branched chain amino acids (Kanzaki et al., 1969; Koike & Koike, 1976). Branched chain 2-oxo acids can act as substrates for OGDC and form acyldihydrolipoyl thioesters. Inactivation of OGDC is observed as these acyl groups are only very slowly transferred to CoA. This covalent modification of OGDC may be a major cause of the toxaemia associated with Maple Syrup Urine Disease (Jackson & Singer, 1983).

There have been reports of micromolar levels of Ca^{2+} stimulating OGDC by decreasing the Km for 2-oxoglutarate in a manner independent to that of adenine nucleotides in all vertebrate tissues studied so far but not in OGDC from insect or plant sources or E. coli (McCormack & Denton, 1979; Denton & McCormack, 1980; McCormack & Denton, 1981; Roche & Lawlis, 1982). In insect sources such as OGDC from blowfly flight muscle the most important regulatory agent appears to be the ATP/ADP ratio (Hansford, 1972). Two other mitochondrial dehydrogenases

are stimulated by similar concentrations of Ca^{2+}: NAD^{+}-dependent isocitrate dehydrogenase and PDC. In the former case the Ca^{2+} effect on enzymic activity is direct as with OGDC. In the case of PDC, however activation is achieved by dephosphorylation of the E1α component of PDC by a specific PDC phosphatase whose activity is stimulated by Ca^{2+}. The fact that the activities of three important mitochondrial dehydrogenases are all affected by a similar concentration of Ca^{2+}, points to a regulatory role for this ion. It has been suggested, that Ca^{2+} is, perhaps, the means whereby extrinsic agents, such as hormones, could regulate intramitochondrial metabolism (Murphy et al., 1980; McCormack & Denton, 1981). Denton et al. (1980) predicted that the intramitochondrial [Ca^{2+}] was in the range 0.1-2.0 uM which is the correct range for a regulatory effect. However other workers have reported concentrations of 30 uM and above which would completely saturate the Ca^{2+} binding sites on these enzymes (Williamson & Cooper, 1980). The concentration of free Ca^{2+} inside the mitochondria must be determined before any role involving Ca^{2+} in regulation can be proposed. If Ca^{2+} is a regulatory factor then the possibility of a mitochondrial calmodulin-like protein must be considered.

An alternative regulatory mechanism exists in E. coli. In this organism OGDC is an inducible enzyme not synthesised during anaerobic growth. During growth on carbon sources requiring terminal oxidation, e.g. glutamate, OGDC is synthesised, but in cells grown on lactate or glucose, OGDC is not synthesised until substantial levels of metabolite have accumulated. Physiological induction of OGDC synthesis may be brought about by 2-oxoglutarate (Amarasingham & Davis, 1965). OGDC is also inducible in Bacillus subtilis where levels increase at

the end of the exponential growth phase because a functional citric acid cycle is required for sporulation. Again 2-oxoglutarate or glutamate, which is formed from 2-oxoglutarate by transamination, is thought to be the agent responsible for induction (Ohne, 1975).

1.13 AIMS OF PROJECT

From the preceeding sections it can be seen that in comparison to the work carried out regarding the structure, function and regulation of OGDC, few studies have addressed the problem of biosynthesis of this multienzyme complex.

The aims of this project, therefore, were to study the biosynthesis of the component enzymes of OGDC in mammalian cell lines, to investigate the possible existence of larger M_r precursors for the individual polypeptides and to obtain data relating to the import of these polypeptides into mitochondria.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS AND BIOCHEMICALS

Benzamidine-HCl, PMSF, 1,10-phenanthroline, leupeptin, DOC, Tween 20, 2,4-DNP, digitonin, TEMED, Coomassie Brilliant Blue R-250, pyruvic acid (monosodium salt), 2-oxoglutaric acid (monosodium salt), NAD^+, TPP, NEM, antifoam A concentrate, iodoacetamide, MOPS, EGTA, DTNB, acetyl-CoA, oxaloacetic acid and malic acid were purchased from Sigma Chemical Co., Poole, Dorset, U.K..

DMSO, DTT, PPO, toluene and sucrose came from Koch-Light Laboratories Ltd., Colnbrook, Berks., U.K..

Tris, CoA (trilithium salt) and ATP were the products of Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K..

Acrylamide, N,N'-methylenebisacrylamide, EDTA (disodium salt), SDS, Folin Ciocalteu's Phenol reagent and L-cysteine-HCl were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K..

Polyethylene glycol 6000 was purchased from Serva, Heidelberg, West Germany. Iodogen™ came from Pierce and Warriner (U.K.) Ltd., Chester, U.K.. Triton X-100 was bought from Rohm and Haas (U.K.) Ltd., Croydon, U.K.. Pyronin Y was obtained from George T. Gurr Ltd., London SW6, U.K.. 2-mercaptoethanol was supplied by Riedel-de-Haën, via A. & J. Beveridge Ltd., Edinburgh, U.K.. Salicylate (sodium salt or free acid) came from Aldrich Chemical Co., Gillingham, Dorset, U.K.. FCCP was the gift of Dr P.G. Heytler, E.I. Du Pont De Nemours and Co. (Inc.), Delaware, U.S.A..

All other chemicals were of analytical grade or of the highest available purity.

2.1.2 RADIOCHEMICALS

N-[Et-2-^{3}H]Ethylmaleimide (50 Ci/mmol) was purchased from New England Nuclear, Southampton, U.K..

L-[^{35}S]methionine (approx. 1200 Ci/mmol) was obtained from Amersham International p.l.c., Bucks., U.K..

Na^{125}I, carrier free, came from the Western Infirmary, Glasgow, U.K..

2.1.3 PROTEINS AND ENZYMES

The following commercial enzyme and protein preparations were obtained from Sigma Chemical Co., Poole, Dorset, U.K.:-

citrate synthase (EC 4.1.3.7) from porcine heart

fumarase (EC 4.2.1.2) from porcine heart

TPCK-treated trypsin (EC 3.4.21.4) from bovine pancreas

β-galactosidase (EC 3.2.1.23) from Escherichia coli

protein A from Staphylococcus aureus, Cowan I strain

bovine serum albumin.

Diaphorase (lipoamide dehydrogenase) (EC 1.8.1.4) from porcine heart was purchased from Boehringer Corporation (London) Ltd., Sussex, U.K..

Electrophoresis calibration kit for low M_r proteins came from Pharmacia (G.B.) Ltd., Milton Keynes, Bucks., U.K..

2.1.4 CHROMATOGRAPHY MATERIAL

Sepharose CL-2B, Sephadex G-25, DEAE-Sephacel and phenyl-Sepharose were the products of Pharmacia (G.B.) Ltd., Milton Keynes, Bucks., U.K..

Procion Red A and Cibacron Blue A dye matrix column materials were purchased from Amicon Ltd., Scientific Systems Division, Upper Mill, Stonehouse, Gloucestershire, U.K..

2.1.5 CELL CULTURE MATERIALS

Glasgow-modified Eagle's medium, L-glutamine (200mM), PK-15, NBL-1 and BRL cell lines were purchased from Flow Laboratories Ltd., Irvine, Ayrshire, U.K..

Glasgow-modified Eagle's medium without L-methionine and L-glutamine, myo-clone foetal calf serum, newborn calf serum, MEM non essential amino acids (100x) and Nunc, sterile Roux flasks and petri-dishes were obtained from Gibco Europe Ltd., Paisley, Renfrewshire, U.K..

The following sterile solutions and sterile glassware were supplied by the staff of the tissue culture unit associated with this department:-

Trypsin

trypsin	15.00g
trisodium citrate	17.76g
NaCl	36.90g
phenol red 1% (w/v)	9.00ml
distilled water to	6.00l
pH	7.8

Sterilise - millipore filtration. Store at -20^{o}C

Versene

NaCl	80.00g
KCl	2.00g
Na_2HPO_4	11.50g
KH_2PO_4	2.00g
versene (EDTA)	2.00g
phenol red 1% (w/v)	15.00ml
distilled water to	10.00l

Sterilise - autoclave. Store at room temperature.

Sodium bicarbonate

$NaHCO_3$	56.00g
phenol red 1% (w/v)	1.5ml
distilled water to	1.0l

Sterilise - millipore filtration. Store at room temperature.

Penicillin/Streptomycin

penicillin	10000000.00U
streptomycin	10.00g
distilled water to	1.0l

Sterilise - millipore filtration. Store at -20°C.

2.1.6 PHOTOGRAPHIC MATERIALS

X-Omat S and XAR-5 X-ray films, Kodak FX-40 liquid fixer and Kodak LX-24 X-ray developer were purchased from Kodak Ltd., Manchester, U.K..

2.1.7 ANIMALS

New Zealand White rabbits and 150-350g female rats (Albino Wistar strain) were provided by the departmental animal house. Bovine hearts were obtained from Glasgow Abbatoir, Duke Street, Glasgow or from Paisley Abbatoir, Sandyford Road, Paisley. The hearts were removed from the animals within one hour of slaughter, chilled immediately on

ice and were used in the laboratory within two hours of slaughter.

2.1.8 MISCELLANEOUS

Freund's Adjuvants (complete and incomplete) were obtained from Difco Laboratories, West Moseley, Sussex, U.K..

Normal rabbit serum came from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, U.K..

Heat-inactivated horse serum was purchased from Gibco Europe Ltd., Paisley, Renfrewshire, U.K..

Nitrocellulose paper (0.45μm pore size) was the product of Schleicher and Schüll, Dassel, West Germany.

Pansorbin (standardised 10% (w/v) formalinised *Staphylococcus aureus* cells) was obtained from Calbiochem-Behring Corp., Bishop Stortford, Herts., U.K..

Nalgene sterilisation filter units, type S (115ml, 0.20 μm pore size) were from Nalge Company, Rochester, New York, U.S.A..

Cronex 'Lightning-Plus' intensifying screens came from Du Pont (U.K.) Ltd., Stevenage, Herts., U.K..

Plast-X cassettes, for exposure of X-ray films, were obtained from Anthony Monk [Eng.] Ltd., Sutton-in-Ashfield, U.K..

2.2 METHODS

2.2.1 ENZYME ASSAYS

a) 2-Oxo Acid Dehydrogenase Complexes

The overall activities of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes were assayed according to the method of Brown & Perham (1976).

Assays were carried out at 30°C in a final volume of 1.0ml of 50mM potassium phosphate buffer, pH 8.0, containing 2.5mM NAD^+, 0.2mM TPP, 1.0mM $MgCl_2$, 0.13mM CoASH, 2.6mM cysteine-HCl and 2.0mM sodium 2-oxoglutarate (or 2.0mM sodium pyruvate). The assay was started by addition of enzyme and the reaction followed by monitoring NADH formation at 340nm.

1 unit of activity (katal) is defined as the amount of enzyme required to catalyse the conversion of one mole of substrate per second under the conditions of the assay.

b) Citrate Synthase

Citrate synthase is assayed, by measuring the appearance of the free sulphydryl group of the released CoA using DTNB (Ellman's reagent), as described by Srere (1969). This reaction is followed at 412nm where the resulting mercaptide ion has a strong absorption (E_{412} 13600).

Assays were carried out at 25°C by adding, in this order, 200µl of 0.5M Tris-HCl, pH 8.0; 200µl of 0.5mM DTNB; 100µl of 3mM acetyl-CoA; distilled water and enzyme to 0.90ml. The change in absorbance at 412nm is measured initially to check for acetyl-CoA deacylase activity. To start the reaction 100µl of 5mM oxaloacetate was added

and the change in absorbance at 412nm monitored once more.

1 unit of activity is defined as the amount of enzyme required to release 1umol CoA per min at 25°C.

c) Fumarase

Fumarase was assayed using a modification of the method of Racker (1950).

The reaction mixture was made up as follows:- 336mg L-malic acid was dissolved in approx. 40ml of 0.1M potassium phosphate buffer, pH7.6. The pH was readjusted to 7.6 with approx. 5ml of 2N NaOH before adjusting to a final volume of 50ml with 0.1M potassium phosphate buffer, pH 7.6.

0.97ml of reaction mixture was placed in a cuvette and the reaction initiated by the addition of up to 30µl of enzyme. The reaction was followed by monitoring the appearance of fumarate, which has a carbon-carbon double bond absorbing at 240nm ($E^{1\mu M}_{1cm\ 240}$ 2.44).

2.2.2 PROTEIN DETERMINATION

Protein was determined by using a modification of the Lowry et al. (1951) procedure as described by Markwell et al. (1976) using bovine serum albumin as a standard.

Stock solutions were prepared as follows:-

Solution A: 2% (w/v) $NaCO_3$, 0.4% (w/v) NaOH, 0.16% (w/v) sodium tartrate and 1% (w/v) SDS

Solution B: 4% (w/v) $CuSO_4.5H_2O$

Solution C: 100 parts A mixed with 1 part B

BSA stock: 1mg/ml BSA stock stored at -20°C

Folin Ciocalteu's phenol reagent: diluted 1:1 (v/v) with distilled water on day of use

A sample volume of 1.0ml containing 10-100µg protein was mixed with 3.0ml of solution C and incubated at room temperature for 10-60 min. Samples were then mixed vigorously with 0.3ml of diluted phenol reagent and incubated at room temperature for a further 45 min before reading the absorbance at 660nm.

2.2.3 GEL FILTRATION

Glass chromatography columns were washed with a dilute detergent solution and rinsed thoroughly with distilled water. A slurry of the gel was poured into the column and packed by allowing a gentle flow of buffer (approx. 5ml/h). Equilibration was performed by pumping 2-3 bed volumes of the required buffer through the column. Samples were pumped through the columns using an LKB 2132 microperpex peristaltic pump and fractions were collected using an LKB 2112 fraction collector.

2.2.4 POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

a) Analytical Gels

SDS-PAGE was carried out using the discontinuous Tris-glycine buffer system of Laemmli (1970).

Stock solutions were prepared as follows:-

Solution A: 3.0M Tris-HCl, pH 8.8, containing 0.25% (v/v) TEMED

Solution B: 28% (w/v) acrylamide containing 0.735% (w/v) N,N'-methylenebisacrylamide, deionised.

Solution C: 0.1M Tris-HCl, pH 6.8, containing 0.8% (w/v) SDS and 0.25% (v/v) TEMED

Solution D: 20% (w/v) SDS

Reservoir buffer: 25mM Tris base containing 192mM glycine and 0.1% (w/v) SDS

Gels were cast using our own, home-made, apparatus in slabs of 19.0cm x 9.5cm x 0.15cm, or, if longer gels were required, using a Bio-rad Protean 16cm apparatus in slabs of 18.0cm x 14.0cm x 0.15cm.

Separating gels of the required percentage were prepared from stock solutions according to the following table:-

	Percentage acrylamide				
	6%	7.5%	10%	12.5%	15%
Solution A	25.0	25.0	25.0	25.0	25.0
Solution B	42.3	52.9	71.5	89.3	105.0
Solution C	1.0	1.0	1.0	1.0	1.0
distilled water	129.2	118.6	100.0	82.2	66.5

(all volumes in the table are in ml)

These solutions were mixed, degassed and polymerisation was initiated by adding 150mg ammonium persulphate. The mixture was degassed again before pouring into the casting apparatus.

Stacking gels were made from stock solutions as follows:-

	Percentage acrylamide	
	6%	3%
Solution B	17.5	8.8
Solution C	10.0	10.0
distilled water	55.0	63.7

(all volumes in the table are in ml)

Polymerisation was initiated with 150mg ammonium persulphate and the stacking gel was allowed to polymerise around a teflon template to allow well formation.

b) Preparative Gels

Preparative polyacrylamide gels were prepared in the same manner as described above but the gel thickness was increased to 0.3cm.

c) Preparation Of Samples For SDS-PAGE And Conditions Of Electrophoresis

Stock solutions were prepared in the following manner:-

Laemmli sample buffer: 62.5mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (w/v) sucrose and 0.01% (w/v) pyronin Y

DTT: 1M DTT. Store at -20°C.

Iodoacetamide: 1M iodoacetamide. Store in dark at 4°C.

Protein samples were heated to 100°C for 2 min in Laemmli sample buffer containing 5% (v/v) 2-mercaptoethanol or 10mM DTT, before being applied to the gel. In addition to the above, iodoacetamide was added to 0.1M in cell extracts and cell subfractions which were to be used for immunoblotting analyses. Electrophoresis was carried out at 50mA until the dye front was within 0.5cm of the bottom of the gel.

d) Staining And Scanning Of Polyacrylamide Gels

Protein bands were visualised by immersing the gels in a solution of (i) 0.04% (w/v) Coomassie Brilliant Blue R-250 in 25% (v/v) isopropanol, 10% (v/v) acetic acid for 16-20h followed by destaining in 10% (v/v) acetic acid.

or (ii) 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v)

methanol, 10% (v/v) acetic acid for 1h followed by destaining in 20% (v/v) methanol, 10% (v/v) acetic acid.

Destained polyacrylamide gels were scanned using an LKB 2202 Ultroscan Laser Densitometer.

e) Determination Of M_r From SDS-Polyacrylamide Gels

M_r determinations were carried out by comparing the mobility of a protein band on SDS-polyacrylamide gels with those of a set of low M_r standard proteins including:- phosphorylase b, 92000; BSA, 67000; ovalbumin, 45000; carbonic anhydrase, 30000; trypsin inhibitor, 21000 and α-lactalbumin, 14000 plus β-galactosidase, 116000.

2.2.5 RADIOLABELLING TECHNIQUES

a) Iodination Of Protein A And M_r Standards

Protein A and M_r standards were iodinated using the solid phase oxidising agent, Iodogen as described by Salacinski et al. (1981).

1mg of Iodogen was dissolved in 0.5ml chloroform and coated onto the surface of a vial by blowing off the chloroform using an air stream. Protein A (1mg) or M_r standards (578µg) dissolved in 0.5ml 20mM Tris-HCl, pH 7.2, containing 0.15M NaCl was added to the vial coated with Iodogen together with 300-500µCi of $Na^{125}I$. Iodination was allowed to proceed for 15 min before the reaction was stopped by removing the sample and applying it to a 8.0cm x 1.3cm Sephadex G-25 column equilibrated in 20mM Tris-HCl, pH 7.2, containing 0.15M NaCl. Fractions containing ^{125}I-labelled protein A were pooled, divided into small aliquots and stored at -20^0C.

b) Labelling Of 2-Oxoglutarate Dehydrogenase With N-[^{3}H]ethylmaleimide

Thiol groups of dissociated 2-oxoglutarate dehydrogenase complex were labelled with N-[^{3}H]ethylmaleimide as follows:-
100µg of purified OGDC was precipitated in 80% (v/v) acetone for several hours at -20°C. The pellet was dissolved in 200µl of 20mM Tris-HCl, pH 7.2, containing 2% (w/v) SDS and incubated with 50µCi (5mM) N-[^{3}H]ethylmaleimide for 30 min at 25°C. Termination of the reaction was achieved by the addition of 2-mercaptoethanol to a final concentration of 5% (v/v). Labelled protein was precipitated in acetone as before. The pellets were washed once in cold acetone, dried, redissolved in 200µl of 2% (w/v) SDS and diluted five fold with 10mM Tris-HCl, pH 7.4, containing 300mM NaCl, 5mM EDTA and 1% (v/v) Triton X-100 (Triton buffer). Radiolabelled protein was stored in small aliquots at -20°C.

2.2.6 DETECTION OF RADIOACTIVITY

a) Measurement Of Gamma Radiation

Radioactivity in samples radiolabelled with ^{125}I was determined by counting in an LKB Wallac 1275 minigamma counter.

b) Liquid Scintillation Spectrometry

Aqueous samples of total volume 0.5ml were made up to 5.0ml with 0.5% (w/v) PPO and 35% (v/v) Triton X-100 in toluene and counted in a Beckman LS6800 liquid scintillation counter.

To facilitate estimation of radioactivity incorporated into protein, 5-50µl samples were placed on Whatman No. 1 filter discs

(2.5cm diam.). Discs were dropped into ice-cold 10% (w/v) TCA, washed a further twice with 10% (w/v) TCA and once with ethanol. All washes were carried out for 30 min at 4°C. Dried discs were placed in scintillation vials containing 5.0ml scintillation fluid and analysed as above.

c) Autoradiography

Autoradiography was employed to visualise ^{125}I-labelled proteins in polyacrlyamide gels or on nitrocellulose paper. The nitrocellulose paper was dried at room temperature and the gels dried under vacuum before exposure to X-Omat-S X-ray film at -70°C with a Cronex 'Lightning Plus' intensifying screen to enhance autoradiographic detection (Laskey & Mills, 1977).

d) Fluorography

Gels for analysis by fluorography were stained in Coomassie blue as described in section 2.2.4 d, or fixed in 25% (v/v) methanol, 10% (v/v) acetic acid before processing with DMSO-PPO as detailed by Bonner & Laskey (1974). Gels were incubated for 3 x 30 min in DMSO, then for 3-4h with 20% (w/v) PPO in DMSO, and washed finally for 1-2h in running water before being dried under vacuum. Thereafter gels were exposed to XAR-5 or X-Omat S X-ray film at -70°C.

Alternatively, gels were processed according to the method of Chamberlain (1979) using the water-soluble fluor, salicylate. Gels were stained or fixed (as above) then washed for 30 min in 2-3 changes of distilled water to remove all traces of acid. Incubation in 1M salicylate for 30 min at room temperature was carried out prior to drying the gel and exposure to X-ray film as described previously.

2.2.7 TISSUE CULTURE

a) Medium

PK-15 cells were routinely grown in Glasgow modified Eagle's medium supplemented with 5% (v/v) foetal calf serum, 1% (v/v) non essential amino acids, 10^5U/1 penicillin, 100mg/1 streptomycin and 2.24g/1 $NaHCO_3$ (normal growth medium-NGM).

NBL-1 and BRL cells were grown in the above medium containing 10% (v/v) foetal calf serum and 10% (v/v) newborn calf serum respectively.

Minus methionine medium (MMM) consists of Glasgow modified Eagle's medium (without L-glutamine or L-methionine) supplemented with 1% (v/v) non essential amino acids, 2mM L-glutamine, 10^5U/1 penicillin, 100mg/1 streptomycin and the appropriate serum type and concentration described above.

Low methionine medium (LMM), used during incorporation of [^{35}S]methionine into cellular protein, was 5% (v/v) normal growth medium in minus methionine medium.

b) Routine Culture Of Cells

Cells were maintained in normal growth medium in plastic Roux bottles at 37°C in an atmosphere of 95% (v/v) air/5% (v/v) CO_2 and were subcultured every 3-4 days.

Monolayers were rinsed twice with versene solution (section 2.1.5), once with trypsin solution (section 2.1.5), then incubated at 37°C with sufficient trypsin to cover the cell monolayer. On release of the cells from the plastic surface, 20ml of NGM was added and the cells disaggregated by gentle passage up and down a sterile pipette. Cells were counted in a haemocytometer and 3-5 x 10^6 cells were

returned to the Roux flask in approx. 50ml NGM.

c) Labelling Of Cells With [^{35}S]methionine

For incorporation of [^{35}S]methionine into protein, cells were introduced into 10cm diam. sterile petri-dishes at a concentration of 3-3.5 x 10^5 cells/ml in 10ml of NGM. Petri-dishes were incubated at 37°C in a humidified incubator containing an atmosphere of 95% (v/v) air/5% (v/v) CO_2 for 24-48h until semi-confluent monolayers were observed. NGM was replaced with 4.0ml of LMM after an initial rinse in the same medium, then incubated for a further 1h to deplete intracellular pools of methionine. After addition of 100-200µCi [^{35}S]methionine to the medium, dishes were incubated at 37°C for either 4h or overnight. Lysis of the cells and preparation of extracts was as described in section 2.2.8 a.

d) Labelling Of Cells With [^{35}S]methionine In The Presence Of Uncouplers Of Oxidative Phosphorylation

Replicate dishes of cells were cultured in NGM as before, including preincubation with LMM for 1h before the addition of 4.0ml of LMM containing uncoupler added from freshly-made stocks of 200mM 2,4-DNP or 1mM FCCP. After 5-15 min at 37°C 200µCi [^{35}S]methionine was introduced asceptically and the incubation continued at 37°C for 4h before preparation of cell lysates (section 2.2.8 a).

e) Pulse-Chase Labelling Of Cells

Cells were labelled for 4h with [^{35}S]methionine in the presence of uncoupler as described above. Radioactive medium was discarded, the cells rinsed in NGM and incubated in 10ml NGM at 37°C for various lengths of time before preparation of cell lysates (section 2.2.8 a).

To monitor the stability of cytoplasmically-located mitochondrial proteins, synthesised in the presence of uncouplers, an alternative protocol was adopted in which the chase was carried out in NGM supplemented with uncoupler at the same concentration as in the 4h incubation with [^{35}S]methionine.

2.2.8 PREPARATION OF CELL EXTRACTS AND SUBFRACTIONS OF CELLS

a) Isolation Of Mitochondria From Tissue Culture Cells

Mitochondria were prepared from cultured cells following the procedure of Attardi & Ching (1979) starting from approx. 2 x 10^8 cells. All procedures were carried out at 4°C.

Cell monolayers were rinsed, scraped into 40ml ice-cold PBS (section 2.2.8 a) and centrifuged for 10 min at 800g. The cells were washed twice in 0.13M NaCl containing 5mM KCl and 1mM $MgCl_2$ before being resuspended in 6.0ml of 10mM Tris-HCl, pH6.7, containing 10mM KCl and 0.15mM $MgCl_2$. Homogenisation was carried out in a Potter-Elvenhjem teflon-glass homogeniser at half maximal speed for 3 strokes, after which the sample was checked under a light microscope for cell breakage. On addition of 0.25M sucrose the solution was centrifuged at 1500g for 3 min to remove nuclei. Mitochondria were pelleted from the supernatant fluid by centrifugation at 5000g for 10 min. Pellets were resuspended in 2ml 10mM Tris-HCl, pH 6.7, containing 0.15mM $MgCl_2$ and 0.25M sucrose. The last two centrifugation steps were repeated. Remaining mitochondria were resuspended in 1.0ml of 10mM Tris-acetate, pH 7.0, containing 0.25M sucrose and the protein concentration was determined by the modified Lowry procedure (section 2.2.2).

b) Fractionation Of Cells Using Digitonin

Digitonin fractionation was used to prepare soluble and particulate fractions of cells using a modification (Mori et al., 1981a) of the method of Zuurendonk & Tager (1974).

Cells were grown in 10cm diam. petri-dishes as described in section 2.2.7 b until semi-confluent. These cells were washed in PBS, scraped off into 0.5ml 20mM K^+Hepes, pH 7.4, containing 0.25M sucrose and 3mM EDTA then resuspended to a single cell suspension. Protein concentration was determined as in section 2.2.2 before cells from one petri-dish were incubated with 0.25-10.0mg digitonin per ml cell suspension for 2 min on ice. Centrifugation for 1 min at 14000g in an MSE Microcentaur microfuge separated the soluble and particulate fractions. Soluble fractions were assayed for citrate synthase (section 2.2.1 b) and fumarase (section 2.2.1. c) activities in order to determine the concentration of digitonin required to break open the highest percentage of cells without disrupting the mitochondria.

In the case of [^{35}S]methionine-labelled cells, monolayers were washed with PBS and the cells scraped into the above buffer supplemented with 1mM benzamidine-HCl and 1mM 1,10-phenanthroline. Digitonin was added to a previously defined concentration and the cytosolic and particulate fractions separated as described above. The particulate fractions were suspended in 100mM Tris-HCl, pH 8.2, containing 100mM KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100, 0.5% (w/v) SDS and 1.0% (w/v) DOC (3D-TKM), and the cytosolic fractions diluted with an equal volume of two times concentrated 3D-TKM. Both fractions were centrifuged at 24000g for 30 min and the clear supernatant fractions

utilised immediately for immune precipitation (section 2.2.9) or stored at -70°C.

c) Preparation Of Cell Lysates From [^{35}S]methionine-Labelled Cells

The following standard solutions were prepared and sterilised by passage through a Nalgene filter (pore size, 0.20μm):-

PBS: section 2.2.8.a

Triton-TKM: 100mM Tris-HCl, pH 8.2, containing 100mM KCl, 5mM $MgCl_2$ and 1% (v/v) Triton X-100

3D-Lysis buffer: 100mM Tris-HCl, pH 8.2, containing 100mM KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100, 1% (w/v) SDS and 2% (w/v) DOC

All solutions were supplemented with 1mM benzamidine-HCl and 1mM 1,10-phenanthroline immediately before use. All procedures were carried out at 4°C. Cell monolayers were washed three times in ice-cold PBS before harvesting into 1.0-1.5ml Triton-TKM. Nuclei were removed by centrifugation at 1000g for 5 min. The supernatant fraction was diluted 1:1 (v/v) with 3D-Lysis buffer. Samples were centrifuged at 24000g for 30 min to remove any remaining cellular debris. Lysates were used immediately for immune precipitation or stored at -70°C.

b) Preparation Of Rat Liver Mitochondria

Mitochondria from rat liver were prepared according to Chance & Hagihara (1963). The following isolation medium was used throughout the preparation:-

2mM MOPS, pH 7.2, containing 0.225M mannitol, 0.075M sucrose and 0.4mM EDTA.

All procedures were carried out at 4°C.

Starved female rats of the Wistar strain (150-350g) were killed by cervical dislocation. Livers removed from these animals were rinsed twice in isolation medium and chopped finely using scissors. Homogenisation was carried out in a Potter-Elvenhjem homogeniser with a loose-fitting teflon pestle for 2 passes at maximal speed to facilitate tissue breakdown followed by 5 passes with a tight-fitting teflon pestle (setting 5, clearance 0.006-0.008 in) to cause cell disruption. The homogenate was centrifuged at 800g for 7 min to remove the nuclei, red blood cells and cellular debris. Mitochondria were collected from the supernatant fraction by centrifugation at 6500g for 15 min. The pellets were washed twice in half the initial volume of isolation medium and again mitochondria were collected by centrifugation as previously described. Mitochondria were resuspended in isolation medium at a final concentration of approx. 20mg/ml.

Isolated mitochondria were purified on a discontinuous sucrose density gradient made up as follows:-

4.0ml	54% (w/w) sucrose in 10mM Tris-HCl, pH 7.5
10.0ml	45% (w/w) sucrose in 10mM Tris-HCl, pH 7.5
10.0ml	39% (w/w) sucrose in 10mM Tris-HCl, pH 7.5
6.0ml	20% (w/w) sucrose in 10mM Tris-HCl, pH 7.5

The gradients were centrifuged at 49000g for 2h in a Beckman SW27 rotor.

After centrifugation 3 bands were visible: the upper band of density 1.16g/cm^3 consists principally of microsomes and plasma membrane; the middle band of density 1.19g/cm^3 represents purified mitochondria; the lower band of density 1.22g/cm^3 is composed mainly of lysosomes with some mitochondrial contamination. Mitochondria

harvested from the gradient are diluted slowly in isolation medium and centrifuged at 6500g for 10 min to remove the sucrose and collect the mitochondria.

c) Preparation Of Bovine Heart Mitochondria

Mitochondria were prepared from bovine heart by Miss Anne Phelps in our laboratory, following the general procedure described by Smith (1967).

Bovine hearts obtained from the slaughterhouse were immediately chilled and all subsequent operations were carried out at 4°C. After trimming off fat and connective tissue, the heart was cut into 5cm cubes, 300g of which were passed through a mincer. Minced tissue was placed in 400ml of 10mM Tris-HCl, pH 7.8, containing 0.25M sucrose and the pH was adjusted to 7.5±0.1 using 2M Tris base. After neutralisation, the ground tissue was placed in cheesecloth, squeezed free of sucrose, then resuspended in 400ml of 10mM Tris-HCl, pH7.8, containing 0.25M sucrose, 1mM Tris-succinate and 0.2mM EDTA (sucrose solution). 3ml of 2M Tris base were added to maintain the correct pH and the material was blended at high speed for 15 sec in a Waring blender. A further 3ml of 2M Tris base were added and blending continued for a further 5 sec. The pH of the solution was further adjusted to 7.8 with 2M Tris base and the homogenate was centrifiged at 1000g for 15 min. The supernatant fluid was centrifuged at 5000g for 30 min. Pellets from this spin were washed once or twice more in sucrose solution. Mitochondria were resuspended in sucrose solution at a final protein concentration of 20-40mg/ml.

2.2.9 IMMUNOLOGICAL TECHNIQUES

a) Immune Blotting

This protocol, which follows a modification (Batteiger et al., 1982) of the method of Towbin et al. (1979), permits the immunological detection of proteins following their electrophoretic transfer from polyacrylamide gels to nitrocellulose paper.

A homogeneous 10% (w/v) polyacrylamide gel was used for resolution of the proteins. Transfer of proteins was carried out electrophoretically as described by Towbin et al. (1979) in 25mM Tris base containing 192mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol for either 4h at 400mA or 16-20h at 40mA. Excess binding sites on the nitrocellulose were blocked by incubation for 60 min at room temperature in 20mM Tris-HCl, pH7.2 containing 0.15M NaCl, 0.5% (v/v) Tween 20 and 5mg/ml NaN_3 (washing buffer). This buffer was discarded and the nitrocellulose incubated for 90 min at room temperature in washing buffer supplemented with 0.5% (v/v) heat-inactivated horse serum and antiserum at a final dilution of 1:50 or 1:100. The nitrocellulose was then washed five times in washing buffer over the space of 1h before incubation for 60 min at room temperature in washing buffer containing ^{125}I-labelled protein A (approx. 10^6 c.p.m.). After being washed another five times in 1h with washing buffer, the nitrocellulose was dried and the antigen was located by autoradiography (section 2.2.6 c).

Following autoradiography the nitrocellulose paper could be stained for protein by immersion in 0.1% (w/v) Amido Black, 50% (v/v) methanol, 10% (v/v) acetic acid for 2-3 min followed by destaining in 50% (v/v) methanol, 10% (v/v) acetic acid for 2-5 min.

b) Immune Precipitation Of ^{3}H-labelled 2-Oxoglutarate Dehydrogenase Complex

Immune precipitaion of ^{3}H-labelled OGDC was carried out in a final volume of 100µl containing 2µg (approx. 30000c.p.m.) of the labelled complex in either Triton buffer (section 2.2.5 b) or 3D-TKM (section 2.2.8 b). Samples were incubated with 2-25µl of antiserum for 1h at room temperature followed by 16-20h at 4°C. At this stage 50µl of Pansorbin was added and the incubation continued for 1h at room temperature in order to precipitate the immune complexes. The pellets were washed three times in Triton buffer or 3D-TKM and once in 10mM Tris-HCl, pH 7.4 by resuspending the pellets in the appropriate buffer and then collecting the immune complexes by centrifugation at 14000g for 2 min. Immune complexes were dissociated from the surface of the S. aureus cells by boiling for 5 min in 50µl of Laemmli sample buffer (section 2.2.4 c). A 5µl aliquot was counted for radioactivity and the remainder of the sample was analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography (section 2.2.6 d).

c) Immune Precipitation Of [^{35}S]methionine-labelled Cell Extracts

The composition of the buffer containing the cell extracts was adjusted to equal that of 3D-TKM (section 2.2.8 b)). Aliquots (100-1000µl) of cell extract containing 10-20 x 10^{6}c.p.m. were incubated with 10-50µl of antiserum for 1h at room temperature followed by 16-20h at 4°C. The immune complexes were precipitated using 100µl of Pansorbin and were washed and analysed as described above.

CHAPTER THREE

PURIFICATION OF 2-OXOGLUTARATE DEHYDROGENASE FROM BOVINE HEART

3.1 INTRODUCTION

The initial stages of the project were concerned primarily with isolating the bovine heart 2-oxoglutarate dehydrogenase multienzyme assembly in a homogeneous state. This purified enzyme complex would then be employed to raise specific antisera, necessary for future work on the biosynthesis, import and maturation of the component enzymes of OGDC.

Several earlier purification procedures for the isolation of mammalian OGDC required preliminary purification of large amounts of mitochondria (Ishikawa et al., 1966; Linn et al., 1972; Koike & Koike, 1976; Roche & Cate, 1977). In 1980, Stanley & Perham described a new method allowing the parallel purification of OGDC and PDC from bovine heart. In this method, the multienzyme complexes were isolated from whole tissue extracts solubilised in the non-ionic detergent Triton X-100. The composition and specific activity, (195 nkat/mg) of OGDC purified by this procedure compared favourably with the same complex purified from bovine heart (220 nkat/mg) by Linn et al. (1972), and porcine liver (188 nkat/mg) by Roche & Cate (1977) employing procedures starting from isolated mitochondria. These specific activities were much higher than those reported by Koike & Koike (1976) for OGDC purified from porcine heart mitochondria (88 nkat/mg) and by Linn et al. (1972) for OGDC from bovine kidney (130 nkat/mg). The method described by Stanley & Perham (1980) was rapid and avoided the need for large-scale preparation and stockpiling of mitochondria. Moreover, the yield of multienzyme complex was much improved, being 80-200 fold higher than that obtained by previous procedures (Linn et al., 1972; Roche & Cate, 1977).

However, in our hands, initial attempts at purifying OGDC from bovine heart resulted in a product of lower specific activity and yield than reported for this procedure (Stanley & Perham, 1980). We also observed an pronounced instability of OGDC during later stages of the purification. Therefore, the basic procedure of Stanley & Perham (1980) was modified in an attempt to improve the product.

3.2 METHODS

3.2.1 PURIFICATION OF 2-OXOGLUTARATE DEHYDROGENASE FROM BOVINE HEART

All operations were carried out at 4°C and fresh bovine hearts were always employed as starting material.

Bovine hearts, chilled immediately after slaughter, were trimmed of fat and connective tissue and cut into 2cm cubes. 250g of tissue was blended for 5 min in a Waring blender at maximum speed in 500ml of ice-cold 50mM MOPS, pH 6.8, containing 2.7mM EDTA, 0.1mM DTT, 3% (v/v) Triton X-100, 1mM PMSF, 1mM benzamidine-HCl and silicone antifoam (0.5ml/l). An equal volume of ice-cold 50mM MOPS, pH 7.0, containing 2.7mM EDTA, 0.1mM DTT and 3% (v/v) Triton X-100 was added to the homogenate and the mixture was centrifuged at 10000g for 20 min. The pellets were resuspended in 500ml of 50mM MOPS, pH 6.8, containing 2.7mM EDTA, 0.1mM DTT and 3% (v/v) Triton X-100 before centrifugation as already described. Supernatant fractions from these two centrifugation steps were combined and the pH was adjusted to 6.45 by adding 10% (v/v) acetic acid. The mixture was stirred for 30 min with 0.12 vol. 35% (w/v) PEG before the precipitate was collected by centrifugation at 25000g for 10 min. The precipitate was resuspended

in 400ml of 50mM MOPS, pH 6.8, containing 2.7mM EDTA, 0.1mM DTT, 1% (v/v) Triton X-100, 0.15μM leupeptin and 0.5% (v/v) rat blood serum (RBS). Full resuspension was achieved by sonication at 100W for 3 x 30 sec on 100ml batches using a Dawes Soniprobe. Clarification of the solution was achieved by centrifugation of the sonicate at 25000g for 40 min, followed by filtration of the supernatant fluid through muslin. This filtrate was maintained at pH 7.0 with 0.5M NaOH while $MgCl_2$ was added at a final concentration of 13mM. Sodium phosphate buffer (1M), pH 6.3 was added to a final concentration of 50mM to raise the ionic strength. This step was necessary in order to avoid the formation of a precipitate when the pH was lowered for the second PEG precipitation. The pH was then lowered to 6.45 prior to addition of 0.12 vol. 35% (w/v) PEG. After stirring for 30 min, the PEG precipitate was collected by centrifugation at 25000g for 10 min. Resuspension of the pellet was achieved by standing overnight in 50mM MOPS, pH 6.8, containing 2.7mM EDTA, 0.1mM DTT, 1% (v/v) Triton X-100, 0.15μM leupeptin and 0.5% (v/v) RBS followed by homogenisation in a Potter-Elvenhjem teflon-glass homogeniser. The solution was warmed to approx. 20°C and stirred gently for 1h prior to sonication at 100W for 3 x 30 sec. Following clarification of the sample by centrifugation at 40000g for 60 min the supernatant fraction was warmed to approx. 20°C and stirred gently for a further 1h. The pH of the solution was lowered to 6.4 by addition of 10% (v/v) acetic acid and 0.04 vol. 35% (w/v) PEG was added. This level of PEG was found to precipitate maximal amounts of OGDC while leaving the majority of PDC in the supernatant fraction. After 30 min stirring, the precipitate containing OGDC was collected by centrifugation at 25000g for 10 min.

Precipitated OGDC was resuspended in 20ml of 50mM sodium phosphate buffer, pH 7.0, containing 2.7mM EDTA and 1% (v/v) Triton X-100. This was centrifuged at 25000g for 10 min to remove any insoluble material before applying to a 110cm x 3cm Sepharose CL-2B column equilibrated in 50mM sodium phosphate buffer, pH 7.0, containing 2.7mM EDTA and 1% (v/v) Triton X-100. The column was run at 24ml/h in the same buffer with the collection of 12ml fractions. OGDC was recovered from peak fractions (35-42) by centrifugation at 176000g for 5h in a Beckman Ti60 rotor. The complex was dissolved in 50mM MOPS, pH 7.0, containing 2.7mM EDTA and 0.01% (w/v) NaN_3 and was stored at -20°C in small portions after mixing with 0.33 vol. glycerol.

3.2.2 CHROMATOGRAPHY OF 2-OXOGLUTARATE DEHYDROGENASE USING VARIOUS COLUMN MATERIALS

OGDC, of known protein concentration and enzymic activity, in 0.5ml of 50mM sodium phosphate buffer, pH 7.0, containing 2.7mM EDTA and 1% (v/v) Triton X-100 was mixed with an equal volume of column material equilibrated in the same buffer. This mixture was incubated on ice for 15 min, with occasional shaking, before centrifugation at 1000g for 5 min. The supernatant fraction was assayed for enzymic activity, the column matrix washed with 1.0ml starting buffer and the resulting supernatant fraction assayed again for enzymic activity. These washes were then repeated with the starting buffer containing 0.25M, 0.5M and 1.0M NaCl and the supernatant fractions assayed after each wash. A further wash in 6M urea containing 0.5M NaOH was carried out and the supernatant fraction analysed by SDS-PAGE.

TABLE 3.1 Purification of 2-oxoglutarate dehydrogenase from bovine heart

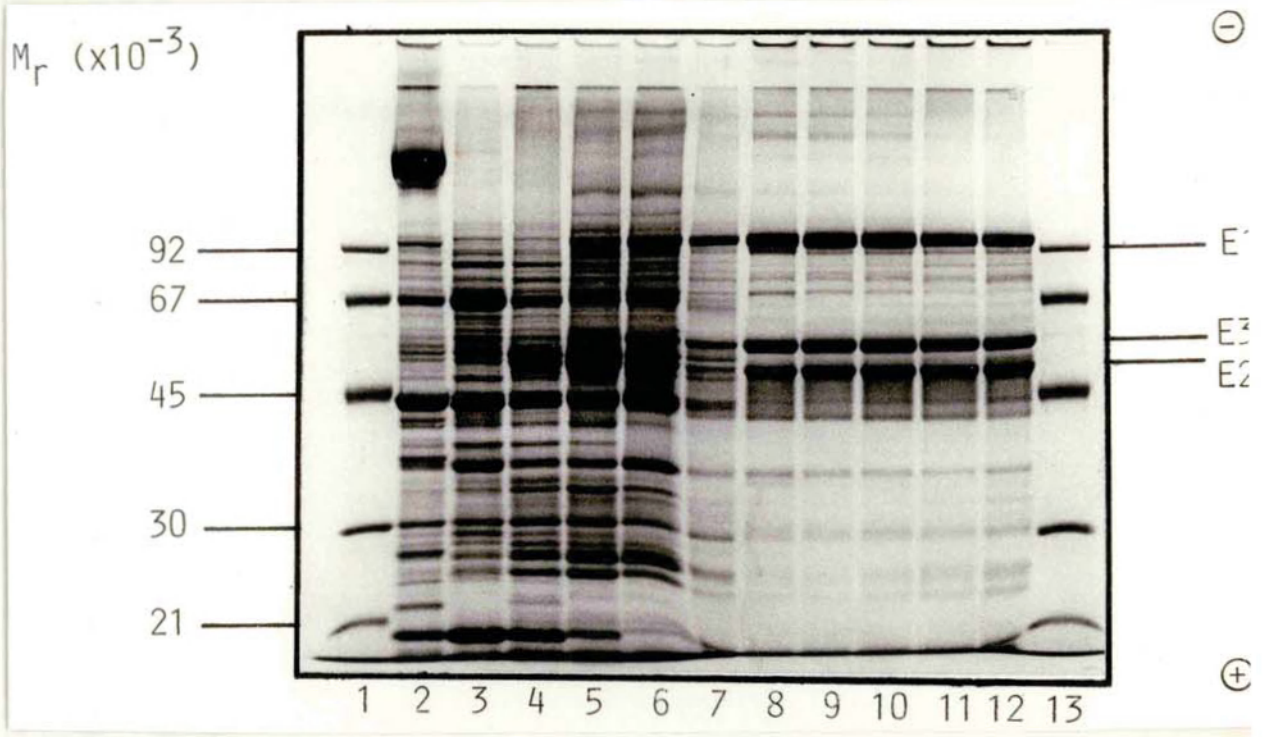
STEP	VOL (ml)	TOTAL PROTEIN (mg)	ENZYME ACTIVITY (nkat)		SPECIFIC ACTIVITY (nkat/mg)		YIELD OGDC (%)
			OGDC	PDC	OGDC	PDC	
Initial homogenate	2000	38950	-	-	-	-	-
1st PEG precipitate	390	3040	18780	20870	6.18	6.86	100
1st PEG precipitate clarified	375	2175	14043	17053	6.46	7.84	75
2nd PEG precipitate	95	922	13320	18297	14.45	19.84	71
2nd PEG precipitate clarified	110	748	11440	16617	15.29	21.21	61
Material loaded onto Sepharose CL-2B	33	264	7677	889	29.08	3.37	41
Concentrated fractions from Sepharose CL-2B (combined)	6	20.4	4065	155	199.26	0.76	22

Purification of 2-oxoglutarate dehydrogenase from 250g of fresh bovine heart is illustrated in the table. Interference from lactate dehydrogenase and NADH oxidase makes it difficult to obtain accurate values for the activities of OGDC and PDC, especially the latter, in the initial homogenate.

FIGURE 3.1 SDS-polyacrylamide gel analysis of the purification of the 2-oxoglutarate dehydrogenase complex from bovine heart.

Fractions from various stages of the purification of OGDC from bovine heart were resolved on a 10% (w/v) SDS-polyacrylamide slab gel and the proteins were visualised by staining with Coomassie blue.

Lanes 1 & 13, M_r standards; lane 2, initial homogenate (50μg); lane 3, 1st extract (50μg); lane 4, 2nd extract (50 μg); lane 5, 1st PEG precipitate, clarified (50μg); lane 6, 2nd PEG precipitate, clarified (50 μg); lane 7, material loaded onto Sepharose CL-2B column (40 μg); lanes 8-12, fractions 35-39 respectively from the Sepharose CL-2B column (15μg).


M_r (x10^{-3})
92
67
45
30
21
1 2 3 4 5 6 7 8 9 10 11 12 13
⊖
⊕

3.3 RESULTS

3.3.1 PROBLEMS ASSOCIATED WITH THE PURIFICATION OF 2-OXOGLUTARATE DEHYDROGENASE FROM BOVINE HEART

A summary of the purification resulting in a product with the highest specific activity and yield obtained is shown in Table 3.1 and SDS-polyacrylamide gel profiles of fractions from various stages of a purification are illustrated in Fig. 3.1.

Purified OGDC is composed of three protein bands with M_r values, determined from SDS-PAGE as, 103500 $\pm$ 1000, 54500 $\pm$ 1000 and 48500 $\pm$ 1000 respectively. These values agree well with those already observed for OGDC isolated from bovine heart and have already been ascribed to the activities of E1, E3 and E2 respectively (Linn, 1971; Stanley & Perham, 1980).

The percentage purity of OGDC fractions from different preparations varied between 75-95% as determined by densitometric scanning of Coomassie blue stained SDS-polyacrylamide gels (OGDC fractions seen in Fig. 3.1 are approx. 75-85% pure; OGDC fractions in Fig. 3.3 are >90% pure).

Inclusion of RBS and leupeptin in the buffers during the later stages of the preparation further protected the complex against endogenous proteases, leading to considerable improvement in overall yield. However this improved yield, 4-16 μkat/kg, was still much lower than that reported, 73 μkat/kg, by the original authors of this method (Stanley & Perham, 1980).

The main steps, during which enzymic activity was lost in the preparation, were in clarification of the PEG precipitates. Losses of up to 30-40% were observed at this stage. On occasions, the pellets

FIGURE 3.2 Gel filtration of partially purified 2-oxoglutarate dehydrogenase complex on Sepharose CL-2B.

Partially purified OGDC (80mg:8.5ml) of specific activity, 25.6 nkat/mg OGDC and 0.8 nkat/mg PDC, was loaded onto a Sepharose CL-2B column (110cm x 3cm) at 24ml/h in 50mM sodium phosphate buffer, pH 7.0, containing 2.7mM EDTA and 1% (v/v) Triton X-100. The column was eluted at 24ml/hr in the same buffer. Fractions of 12.0ml were collected, assayed for overall OGDC (o) and PDC (●) activities as described in section 2.2.1.

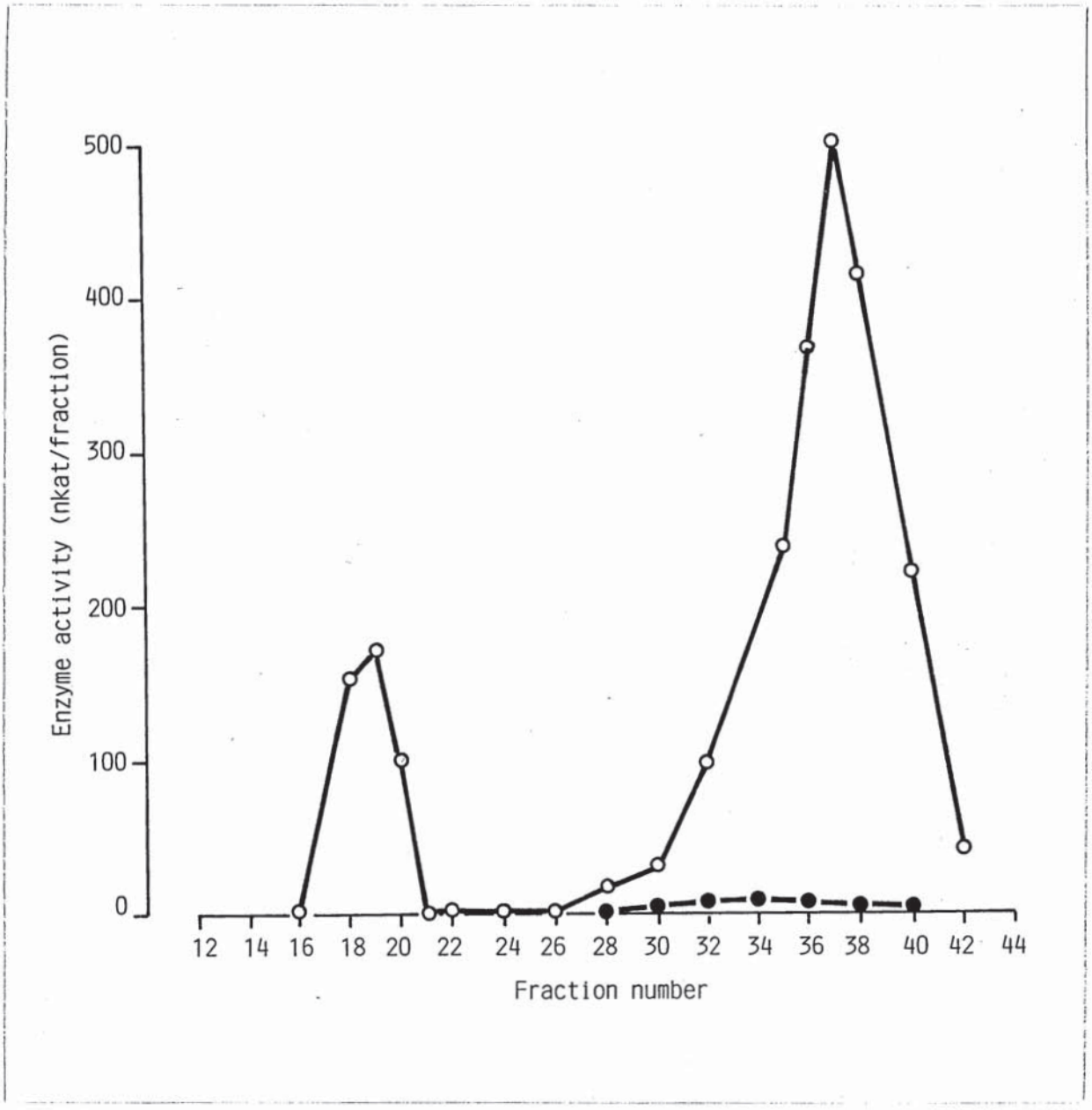
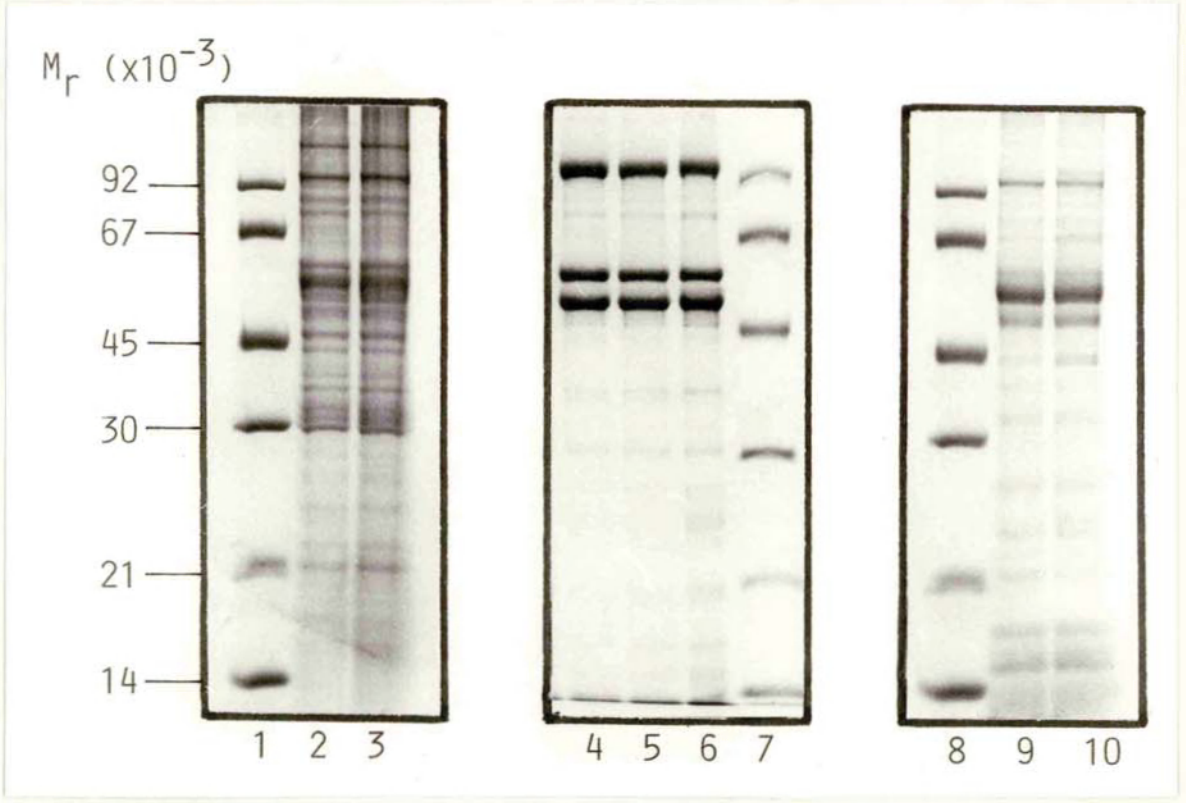

500
400
300
200
100
0
Enzyme activity (nkat/fraction)
12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Fraction number

FIGURE 3.3 SDS-polyacrylamide gel analysis of fractions from the Sepharose CL-2B column.

Peak fractions from the Sepharose CL-2B column (Fig. 3.2) were subjected to SDS-polyacrylamide gel electrophoresis in 10 % (w/v) slab gels and the proteins were detected by staining with Coomassie blue.

Lanes 1,7 & 8, M_r standards; lane 2, fraction 18 (50µg); lane 3, fraction 19 (50µg); lane 4, fraction 36 (15µg); lane 5, fraction 37 (15µg); lane 6, fraction 38 (15µg); lane 9, fraction 42 (10µg); lane 10, fraction 43 (10µg).


M_r (x10^{-3})
92
67
45
30
21
14
1 2 3
4 5 6 7
8 9 10

from these centrifugation steps were re-extracted in 50mM MOPS, pH 6.8, containing 2.7mM EDTA, 0.1mM DTT, 1% (v/v) Triton X-100, 0.15μM leupeptin and 0.5% (v/v) RBS. Although this resulted in a 10-20% higher recovery at this stage, losses of 10-20% of total enzyme activity were still common. Increasing the Triton X-100 content of the re-extraction buffer to 3% (v/v), equivalent to that in the initial extraction buffer, had no effect.

Losses of total enzymic activity were also observed on the Sepharose CL-2B column. Recovery of enzymic activity from this column was approx. 50% on average. Analysis of the activity profile of OGDC eluting from the Sepharose CL-2B column revealed two peaks of enzymatic activity (Fig. 3.2). Fractions from the first peak of enzymic activity were opalescent and SDS-PAGE analysis showed that these fractions contained many proteins exhibiting a broad range of subunit M_r values (Fig.3.3). A similar peak, eluting at the void volume, is found with preparations of PDC from bovine heart (O. L. De Marcucci, personal communication), and Bacillus subtilis (Hodgson, 1983). This fraction from bovine heart, was seen to have a vesicular structure when examined under the electron microscope (Hodgson, 1983). One possibility is that this is a proteolipid structure formed from the cell membranes during sonication.

Fractions from the second major peak of enzymic activity contained pure OGDC (Fig. 3.3) which was concentrated as described previously. On occasions, pooled fractions of OGDC were passed down the Sepharose CL-2B column a second time to further increase the purity of the complex.

FIGURE 3.4 2-oxoglutarate dehydrogenase fractions from the Sepharose CL-2B column are contaminated with a 50000 M_r protein.

Peak fractions from the Sepharose CL-2B column were subjected to SDS-polyacrylamide gel electrophoresis on a 10% (w/v) slab gel. Protein bands were detected by staining with Coomassie blue.

Lanes 1 & 8, M_r standards; lanes 2-7, fractions 35-40 respectively from the Sepharose CL-2B column (15µg) (see Fig. 3.2).

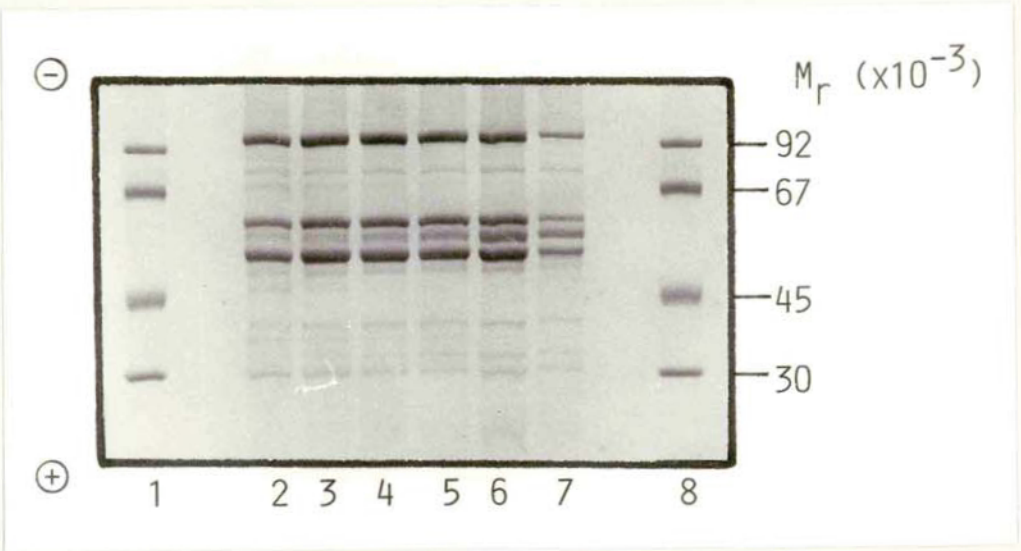
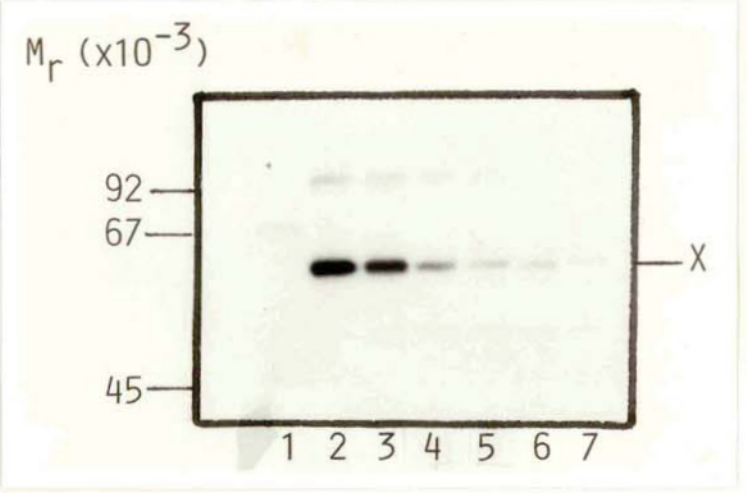

⊖
⊕
M_r (x10^{-3})
92
67
45
30
1
2 3 4 5 6 7
8

FIGURE 3.5 Reactivity of anti-X serum with the 50000 M_r contaminant of purified 2-oxoglutarate dehydrogenase complex.

Peak fractions from the Sepharose CL-2B column were separated into component polypeptides on a 10% (w/v) SDS-polyacrylamide slab gel. One half of the duplicate gel was stained with Coomassie blue (see Fig. 3.4). Polypeptides on the other half were electrophoretically transferred to nitrocellulose paper and the replica was processed for detection of antigenic polypeptides using a 1:50 dilution of anti-X serum.
Lanes 1 & 8, M_r standards; lanes 2-7, fractions 35-40 respectively from the Sepharose CL-2B column (15µg)(see Fig. 3.4).


M_r (x10^{-3})
92
67
45
X
1 2 3 4 5 6 7

3.3.2 CONTAMINATION OF 2-OXOGLUTARATE DEHYDROGENASE WITH A 50000 M_r PROTEIN

Fractions from the Sepharose CL-2B column, immediately following the main peak of enzymic activity, were observed to be brown in colour. When analysed by SDS-PAGE they were found to contain a major component of subunit M_r 50000 (Fig. 3.3). In some preparations, this peak was found to overlap with the peak of enzymic activity. Thus, OGDC fractions from these preparations were contaminated with the 50000 M_r protein (Fig. 3.4).

PDC, which is purified in parallel with OGDC using this procedure, has been found to contain an associated polypeptide of unknown function, known as component X (De Marcucci & Lindsay, 1985), which is similar in M_r to this 50000 M_r contaminant band of OGDC. In order to test whether these proteins were identical or related, antiserum raised against component X (a gift from O. L. De Marcucci) was employed in immune blotting analysis to probe the peak fractions from the Sepharose CL-2B column (Fig. 3.5). By comparing Fig. 3.4 with Fig. 3.5 it can be seen that whereas the amount of the contaminant present in OGDC increases in peak fractions 35-40, the amount of X is highest in fraction 35 and lowest in fraction 40. This infers that these protein species are not related since their distribution would be expected to be parallel were this true. The presence of X in early fractions of the peak from the Sepharose CL-2B column probably reflects contamination of these fractions with PDC. PDC elutes slightly before OGDC from this column and the material loaded onto the column generally contained low levels of PDC which co-precipitated with OGDC during separation of the complexes.

Various column materials were employed in attempts to eliminate

TABLE 3.2 Binding and elution of 2-oxoglutarate dehydrogenase complex to phenyl-sepharose, DEAE-sephacel, Procion Red-A and Cibacron Blue-A dye matrix column materials.

COLUMN MATERIAL	MATERIAL LOADED	INITIAL SUPERNATANT	WASH	0.25M NaCl	0.5M NaCl	1M NaCl	TOTAL % ACTIVITY RECOVERED
phenyl-sepharose	16.1	8.6	3.8	0	0	0	77%
DEAE-sephacel	16.3	0.24	0	5.2	5.0	1.4	73%
Procion Red-A	11.8	0.32	0	0	0	0	3%
Cibacron Blue-A	11.8	0	0	0	0	0	0%

Enzyme activity, in this table, is expressed in nkatal units.

FIGURE 3.6 Elution profile of 2-oxoglutarate dehydrogenase complex from DEAE-Sephacel.

Peak fractions (5.5mg) from the Sepharose CL-2B column were concentrated by ultacentrifugation for 5h at 176000g, resuspended in 4.0ml of 50mM sodium phosphate buffer, pH 7.0, containing 2.7mM EDTA and 1% (v/v) Triton X-100 and loaded onto a 6cm x 1.5cm DEAE-Sephacel column. The column was washed with 35ml of the above buffer, eluted using a 0-0.5M NaCl gradient and washed with a further 10ml of the above buffer, containing 0.5M NaCl. Fractions of 2.5ml were collected, assayed for OGDC activity and monitored for protein content.

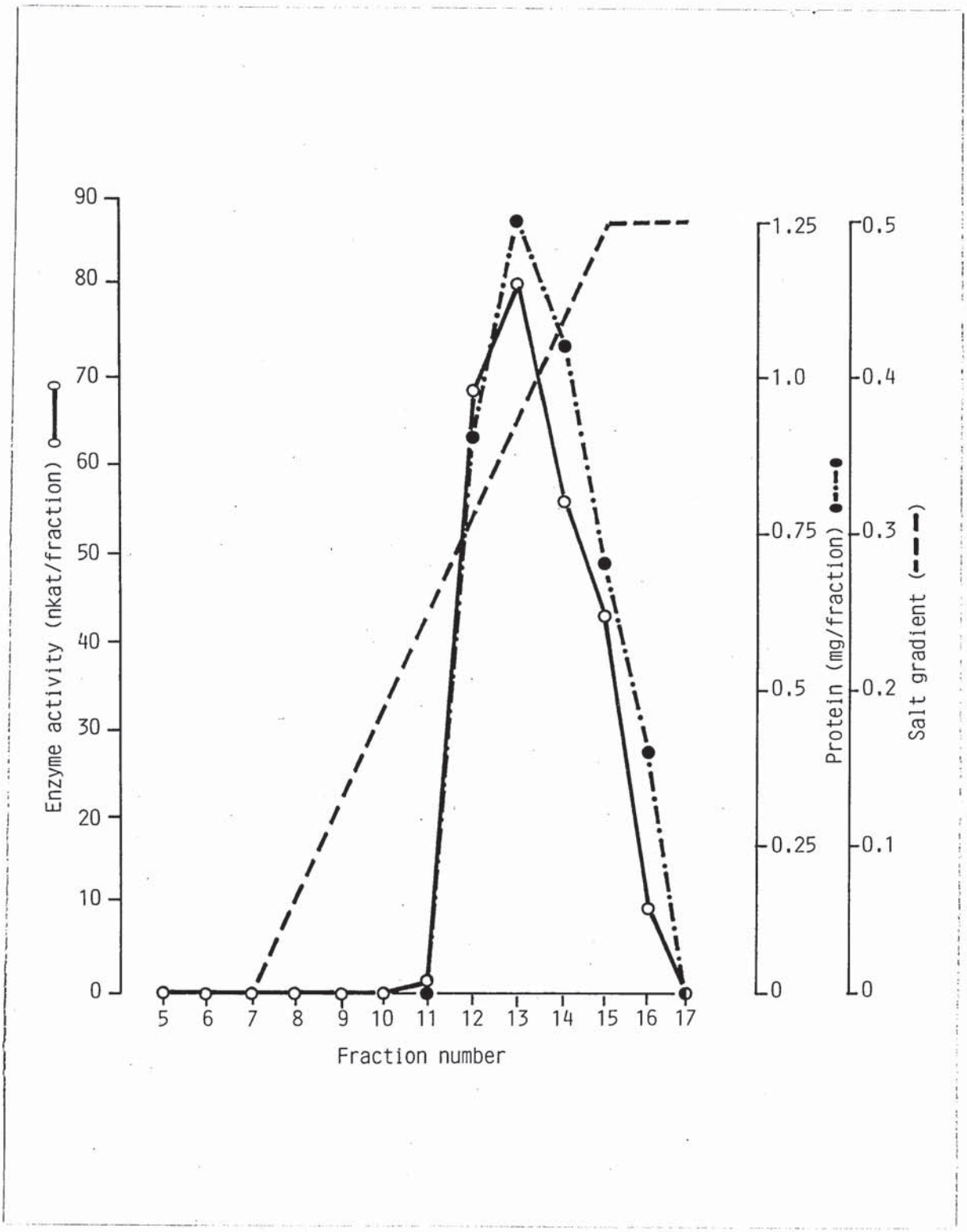
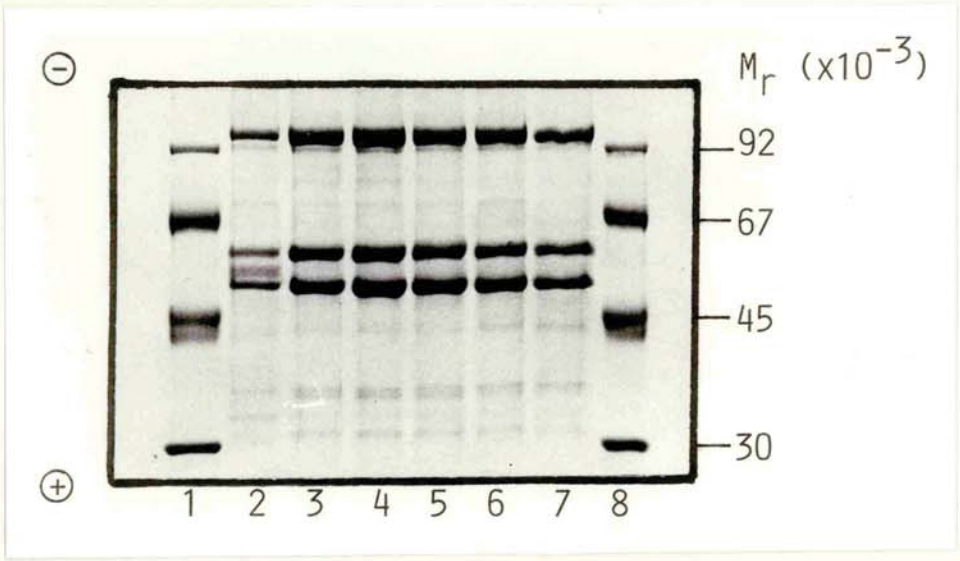

Enzyme activity (nkat/fraction)
90
80
70
60
50
40
30
20
10
0
5 6 7 8 9 10 11 12 13 14 15 16 17
Fraction number
1.25
1.0
0.75
0.5
0.25
0
Protein (mg/fraction)
0.5
0.4
0.3
0.2
0.1
0
Salt gradient

FIGURE 3.7 SDS-polyacrlyamide gel analysis of fractions from the DEAE-Sephacel column.

Material loaded onto the DEAE-Sephacel column and fractions collected from the column were compared by polyacrylamide gel electrophoresis on 10% (w/v) slab gels. Resolved proteins were located by staining with Coomassie blue.

Lanes 1 & 8, M_r standards; lane 2, material loaded onto DEAE-Sephacel (15µg); lanes 3-7, fractions 12-16 respectively from DEAE-Sephacel (15µg) (see Fig. 3.6).


⊖
⊕
M_r (x10^{-3})
92
67
45
30
1 2 3 4 5 6 7 8

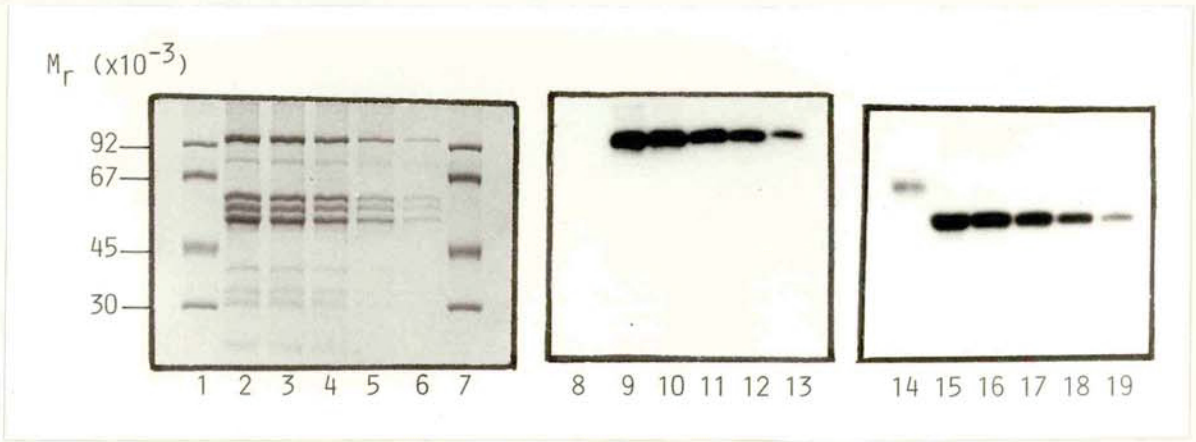
this 50000 M_r contaminant protein including phenyl-Sepharose, DEAE-Sephacel, Procion Red A and Cibacron Blue A dye matrix columns. Initial screening was carried out using the batch method described in section 3.2.2. It was found that OGDC did not bind to phenyl-Sepharose to any significant extent, whereas the binding to Red A and Blue A dye matrix column materials was strong and was not reversed by 1.0M NaCl (Table 3.2) or by 6M urea containing 0.5M NaOH (not shown). These initial experiments indicated that DEAE-Sephacel bound OGDC and that the binding could be reversed by increasing the salt concentration (Table 3.2). Further experiments in which constant amounts of DEAE-Sephacel were incubated with increasing amounts of OGDC were carried out to determine the binding capacity of the gel matrix for OGDC. This was found to be approx. 2mg OGDC per ml gel.

OGDC fractions, containing the 50000 M_r contaminant, were pooled and applied to a 8.0cm x 1.3cm column of DEAE-Sephacel equilibrated in 50mM sodium phosphate buffer, pH 7.0, containing 2.7mM EDTA and 1%(v/v) Triton X-100. The column was washed in the loading buffer and eluted with a linear salt gradient, 0-0.5M NaCl (Fig. 3.6). OGDC eluted in the region 0.30-0.45M NaCl and a 75-90% recovery of protein from this column was observed. Recovery was not increased by washing the column in starting buffer supplemented with 1.0M NaCl. SDS-PAGE of the fractions eluted from DEAE-Sephacel showed that the 50000 M_r contaminant band had been removed (Fig.3.7).

OGDC fractions from Sepharose CL-2B were observed to contain small amounts of PDC. Attempts to resolve the two complexes on DEAE-Sephacel failed as the two enzyme complexes gave a similar elution profile on this column.

FIGURE 3.8 Cross reaction of subunit specific antisera with contaminant bands in purified 2-oxoglutarate dehydrogenase.

Various amounts of purified OGDC were subjected to polyacrylamide gel electrophoresis on a 10% (w/v) SDS-polyacrylamide slab gel. One third of the triplicate gel was stained with Coomassie blue (lanes 1 & 7, M_r standards; lanes 2-6, 20, 15, 10, 5 and 2μg respectively of purified OGDC). Protein bands on the remainder of the gel were electrophoretically transferred to nitrocellulose paper. One half of the nitrocellulose was processed for detection of antigen using anti-E1 serum (lanes 8-13, as lanes 1-6), and the remainder with subunit specific antiserum directed against E2 (lanes 14-19, as lanes 1-6).


M_r (x10^{-3})
92
67
45
30
1 2 3 4 5 6 7
8 9 10 11 12 13
14 15 16 17 18 19

3.3.3 MINOR CONTAMINANTS OF 2-OXOGLUTARATE DEHYDROGENASE ARE NOT IMMUNOLOGICALLY RELATED TO THE COMPONENT ENZYMES OF THE COMPLEX

Protein bands ranging in M_r from 100000 to 20000 were generally observed as minor contaminants of purified OGDC. Subunit specific antisera, raised to the E1 and E2 components of OGDC, as described in section 4.2, were used to probe purified fractions of the complex in order to determine whether these minor bands were the products of proteolysis of E1 or E2 respectively. Fig. 3.8 shows the result of this immune blotting analysis where it can be seen that E1 and E2 subunit specific antisera each react with one band only, corresponding in M_r to the parent antigen. The antisera did not cross react with any of the minor bands which would be expected if these minor components were proteolytic fragments of the original subunits (De Marcucci *et al.*, 1985a; De Marcucci & Lindsay,1985). It was felt that these minor bands were unlikely to be degradation products of E3 since it had been demonstrated that E3 was resistant to degradation by several proteases including trypsin, elastase and papain (not shown). Indeed later characterisation of E3 antiserum by immune blotting versus purified OGDC revealed only one major band.

Further attempts to remove these contaminant bands involved washing the multienzyme complex in various detergent and salt solutions but no significant increase in purity was observed.

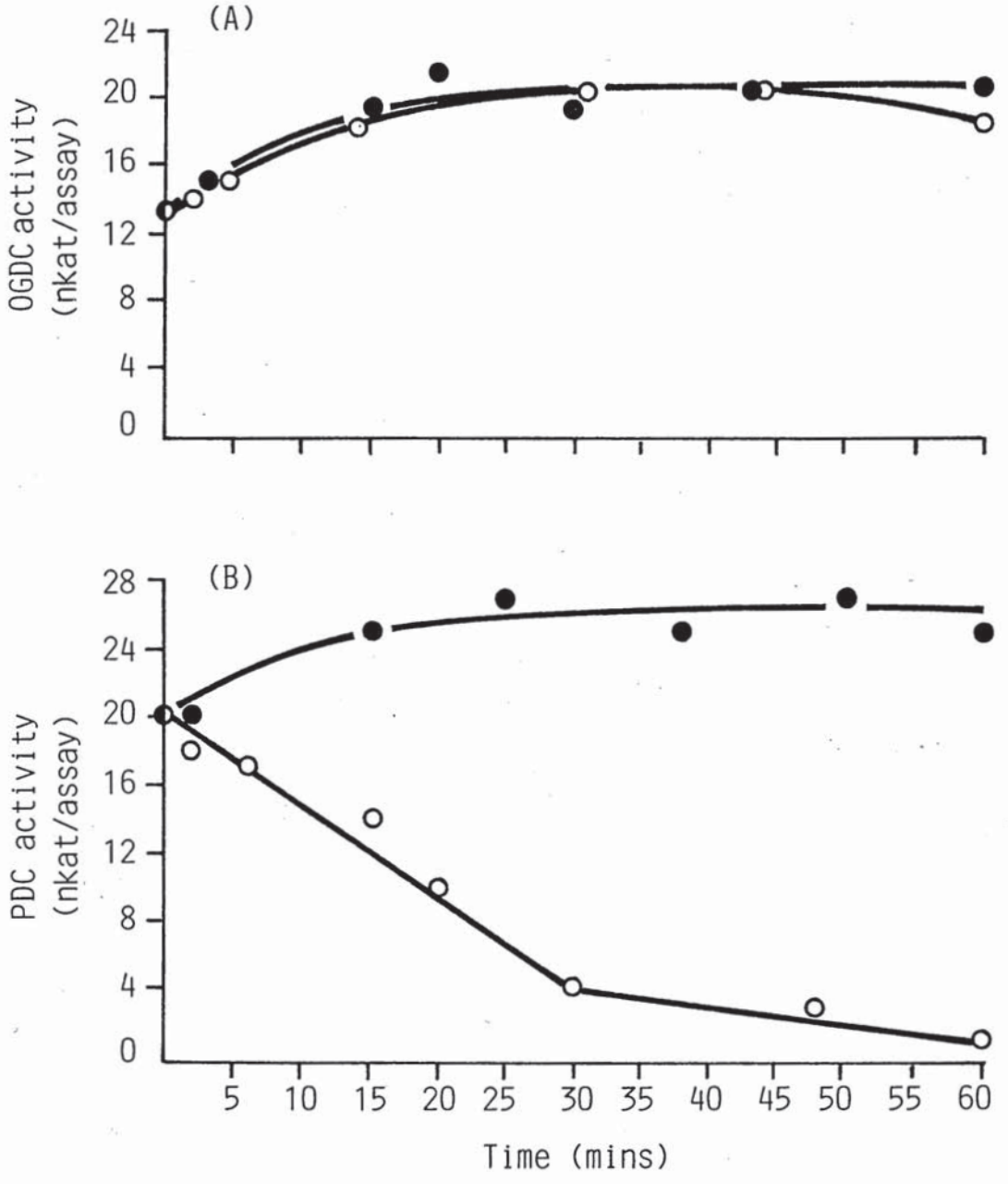
One possible source of contamination of OGDC are ribosomes. As they are similar in size they may elute in the same region as OGDC from the Sepharose CL-2B column, in this case they would also spin down during the concentration of OGDC. It may be possible to eliminate this potential source of contamination by treatment of fractions with ribonuclease, causing disassembly of the ribosome, before collecting

FIGURE 3.9 Effect of ATP on the enzymic activities of the 2-oxoglutarate dehydrogenase complex and the pyruvate dehydrogenase complex.

OGDC (80μg) or PDC (40μg) were incubated at 30°C in 20mM potassium phosphate buffer, pH7.2, containing 3mM $MgCl_2$, 0.2mM DTT, plus or minus 0.2mM ATP, in a final volume of 200μl. At various time intervals, 20μl aliquots were withdrawn from the incubation mix and assayed for enzymic activity.

(A) OGDC activity measured in the presence (o) or absence (●) of ATP.

(B) PDC activity measured in the presence (o) or absence (●) of ATP.


(A)
OGDC activity
(nkat/assay)
24
20
16
12
8
4
0
(B)
PDC activity
(nkat/assay)
28
24
20
16
12
8
4
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (mins)

OGDC by centrifugation.

3.3.4 EFFECT OF ATP ON THE ACTIVITY OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

The effect of ATP on the enzymic activity of OGDC was tested. It is known that the activities of PDC and BCDC are regulated by alteration of the phosphorylation state of these complexes (Reed & Yeaman, 1985). The E1α subunits of these complexes are phosphorylated in the presence of ATP by specific kinases with concomitant loss of activity. No role involving covalent modification has been ascribed to ATP in the regulation of OGDC. Incubation of OGDC in the presence of ATP showed no significant increase or decrease in overall complex activity compared with OGDC incubated minus ATP. In contrast, incubation of PDC in the presence of ATP resulted in a large, rapid inhibition of overall activity (Fig.3.9). It seems unlikely that OGDC activity is regulated by covalent modification involving ATP. The structure of OGDC differs significantly from that of the other 2-oxo acid dehydrogenase complexes. PDC and BCDC both have an $\alpha_2\beta_2$ type of E1 subunit whereas the E1 component of OGDC has an α_2 subunit arrangement. In addition, when isolated mitochondria were incubated with ^{32}P, only two radioactive bands were seen after the proteins were separated by SDS-PAGE and analysed by autoradiography. The M_r values of these bands corresponded with those of the E1α subunits of PDC and BCDC respectively (Cook *et al.*, 1983).

3.4 DISCUSSION

The modified method, described in this chapter, allowed purification of OGDC which closely resembles that reported by Stanley & Perham (1980) in subunit M_r and specific activity.

Fresh bovine hearts were always employed as starting material as it was found that the total enzymic activity extracted from tissue stored at either -20°C or -70°C was up to 50% less than that obtained from the fresh tissue.

Supplementing buffers with RBS and leupeptin greatly improved the yield of OGDC. Difficulties in the isolation of OGDC and PDC, especially from the gluconeogenic organs, kidney and liver, have already been observed (Linn, 1971; Weiland, 1975; Roche & Cate, 1977). In a study on the instability of OGDC in bovine kidney extracts, Linn (1971) found that the loss of activity was not caused by inactivation of any of the component enzymes of OGDC but was due to limited proteolysis of the E2 subunit which resulted in dissociation of the complex. This disassembly of the complex was responsible for the decrease in overall activity of OGDC. The protein which caused this cleavage, purified approx. 100-fold from bovine kidney, requires a thiol group for activity and shows no susceptibility to PMSF or diisopropyl-phosphofluoridate. It behaves like a protein of M_r 25000-30000 on gel filtration. A similar protease, responsible for inactivation of OGDC and PDC in rat liver extracts, was partially purified by Lynen *et al.* (1978). This enzyme which exhibited an M_r of 21000 was also shown to be a thiol protease which was not affected by PMSF. Activity of this 'inactivase' was inhibited by leupeptin, iodoacetamide and rat serum. This effect of rat serum, attributed to the presence of α_2-

macroglobulin, a potent protease inhibitor, had been reported previously by Weiland (1975). In the purification of OGDC and PDC from porcine liver, Roche & Cate (1977) included rabbit serum in their buffers. Omission of this protective agent resulted in a PDC product which had a lower specific activity, and a lower sedimentation coefficient (55S as opposed to 62S). In addition extra lower M_r bands were observed on SDS-PAGE together with a decrease in the relative intensity of the E2 component. These authors also reported that OGDC was more sensitive than PDC to proteolysis. In preparations carried out even in the presence of RBS, extra low M_r bands were observed, which were thought to be proteolytic products of the original subunits. In view of the result obtained in Fig. 3.8, where the additional lower M_r bands of bovine heart OGDC were shown not to be related to either the E1 or E2 subunits, this conclusion, drawn by Roche & Cate (1977), may not be correct. However, as mentioned earlier, even with leupeptin and RBS present, the yield of enzyme obtained is much less than reported by Stanley & Perham (1980) and the reason for this is unclear.

The variation in percentage purity of OGDC between preparations also remains to be explained. It may be due to variation in the bovine hearts or in the chemicals used to prepare the solutions. In particular it was found that the source of the PEG was important. The most effective PEG, for purposes of purification of OGDC, was produced by Serva, Heidelberg, West Germany.

Investigation of storage conditions revealed that storage at $-20^{\circ}C$ with 0.33 vol glycerol was superior. Fractions were stored in small aliquots, as repeated freezing and thawing led to the appearance of a prominent 43000 M_r protein, which was shown to be related to E2

by immune blotting analysis using anti-E2 serum, and several other minor bands, which were also immunologically related to the component enzymes of OGDC. Under these conditions enzymic activity was preserved for up to 6 months at least.

Overall, this procedure allowed the isolation of OGDC in a sufficiently pure form to allow it to be used for the production of antiserum to whole native OGDC and the E1 and E2 component enzymes of OGDC, as will be described in the following chapter.

CHAPTER FOUR

PREPARATION AND CHARACTERISATION OF ANTISERA

4.1 INTRODUCTION

The experimental approaches which have been employed to investigate the biosynthesis of mitochondrial precursors and their import into the organelle have used highly specific antisera raised against the mature protein with the assumption that cross reaction with the precursor will occur. In the majority of cases this assumption has proved valid. However, it is not unknown for antiserum raised against the mature protein to cross react only weakly or not at all with the precursor molecule. Antiserum raised against apocytochrome c will not cross react with the holocytochrome and vice versa (Hennig & Neupert, 1981). Also antiserum raised to subunit IX of the F_1-ATPase complex reacts only very weakly with the precursor form of this protein (Schmidt *et al.*, 1983b).

Therefore, in order to monitor the molecular events in the biosynthesis, import and maturation of OGDC, it was necessary to obtain high titre, monospecific antisera to native, intact OGDC and subunit specific antisera to the component enzymes of this multiprotein assembly. This chapter deals with the production and characterisation of these antisera.

4.2 METHODS

4.2.1 PREPARATION OF ANTISERUM TO NATIVE INTACT 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

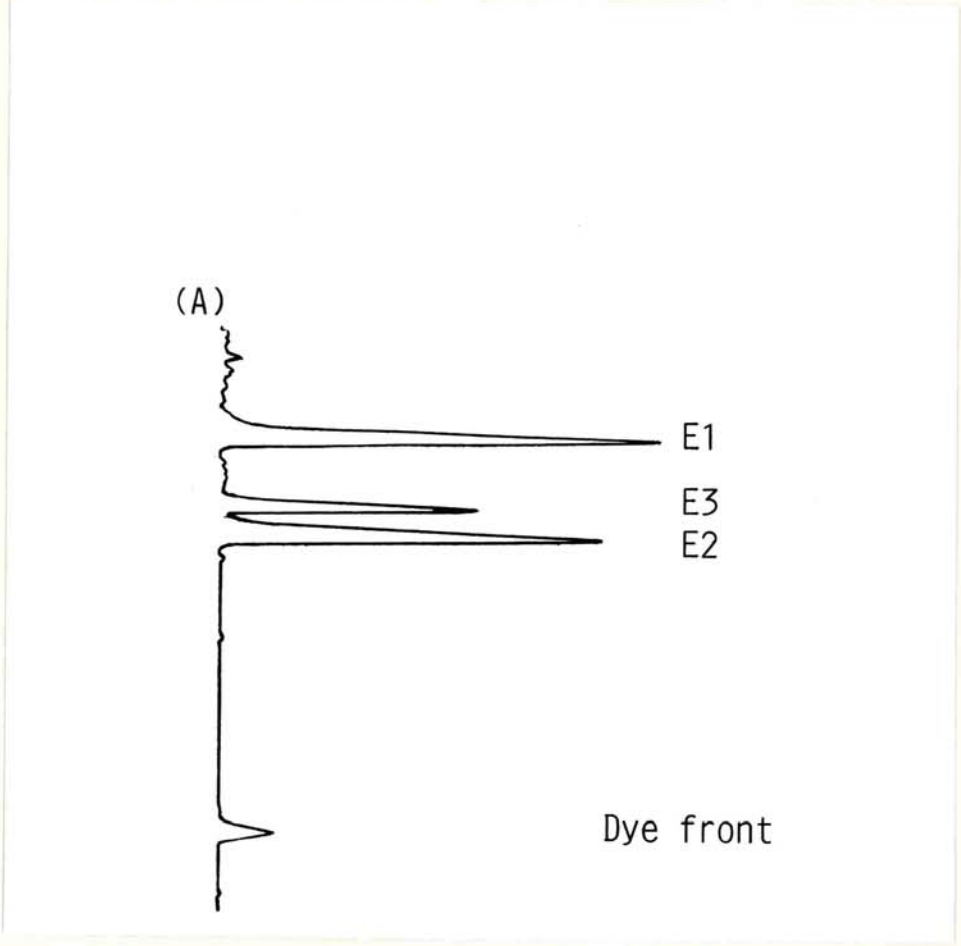
OGDC, purified from bovine heart by the procedure described in section 3.2, was resolved by SDS-PAGE on 10% (w/v) slab gels. Densitometric scanning of Coomassie blue-stained gels allowed the selection of fractions of approx. 95% purity for immunisation of

FIGURE 4.1 Determination of purity of 2-oxoglutarate dehydrogenase complex fractions employed to raise antiserum to the intact, native complex.

OGDC (15ug) was resolved into its native component enzymes by SDS-PAGE on 10% (w/v) slab gels. Following staining of the gel in Coomassie blue, purity was determined by densitometric scanning of the protein bands.

(A) Densitometric trace of protein bands.

(B) Table showing % total area occupied by each peak.

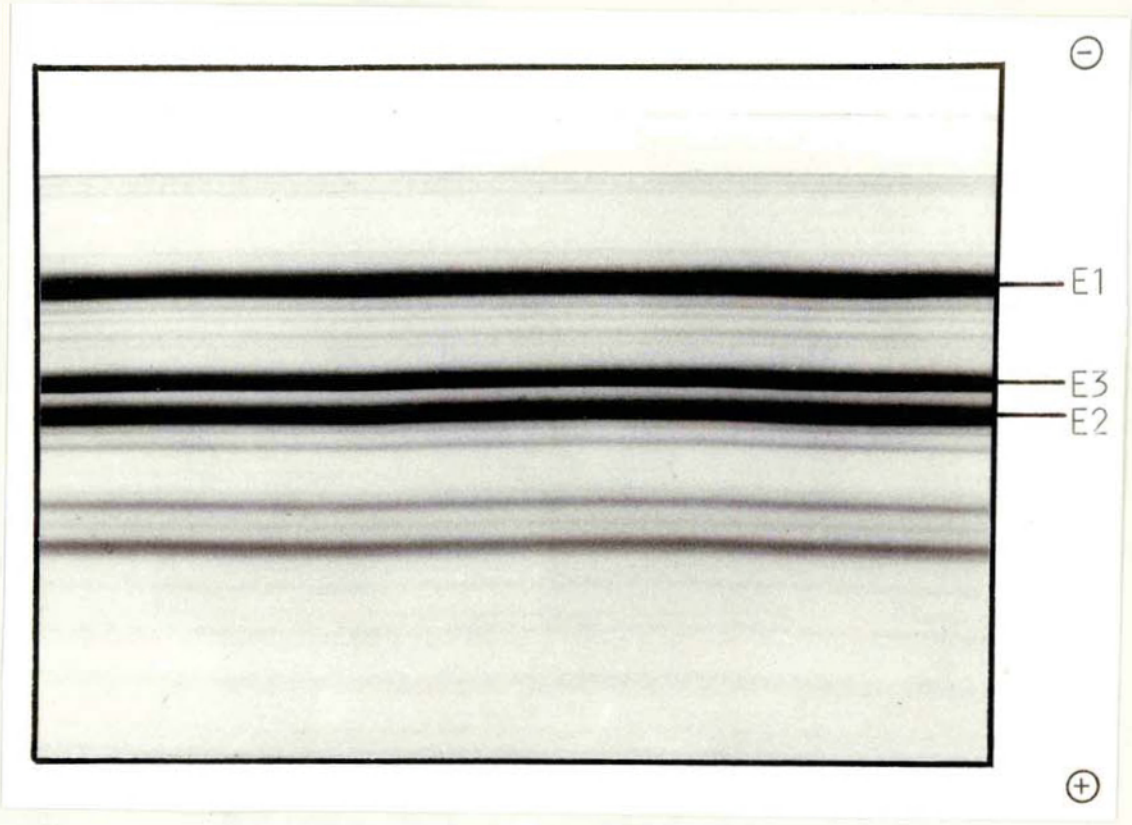


(B)

Protein Band	% Total Area
E1	41
E2	34
E3	19
OGDC	94

FIGURE 4.2 Preparative SDS-polyacrlyamide gel electrophoresis of the 2-oxoglutarate dehydrogenase complex from bovine heart.

Purified OGDC (1.5mg) was resolved into its constituent polypeptides on a 10% (w/v) preparative slab gel. Protein bands were visualised by staining in Coomassie blue, excised from the gel and utilised to raise subunit specific antisera as described in section 4.3.


⊖
E1
E3
E2
⊕

rabbits with native, intact OGDC (Fig. 4.1).

OGDC (1mg) dissolved in 0.5ml of 50mM MOPS, pH 7.0, containing 2.7mM EDTA was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites in the neck and back of a New Zealand White rabbit. Similar amounts of purified enzyme complex were administered at 2-3 week intervals thereafter. Ten days after the 3rd injection, blood was obtained from a marginal ear vein. Additional booster injections were administered at approximately 1 month intervals with 0.5mg of protein mixed with incomplete Freund's adjuvant. The serum was collected, divided into 1.0ml aliquots and stored at -20^{o}C until use.

4.2.2 PREPARATION OF ANTISERA TO THE E1 AND E2 COMPONENT ENZYMES OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

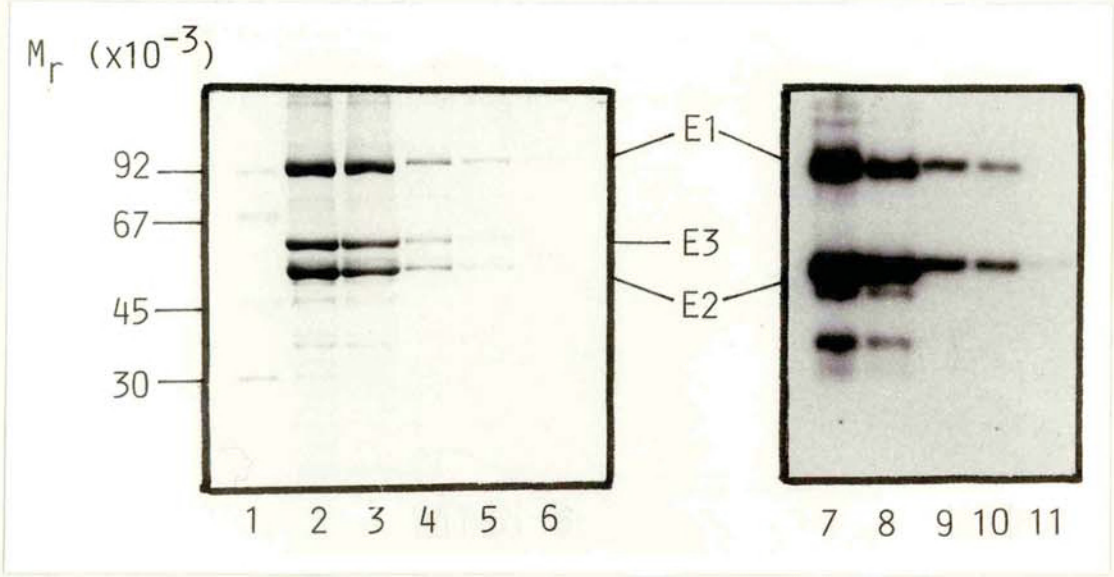
Preparative SDS-PAGE was employed in order to obtain highly purified samples of the individual components of the complex which were necessary for the production of subunit specific antisera. In this way, it was possible to raise highly specific antisera to the E1 and E2 components of OGDC without the necessity for isolating large quantities of enzyme and resorting to the time consuming, conventional procedures for purification of the individual enzymatic activities (Koike & Koike, 1976).

Samples of OGDC of 80-95% purity (approx. 1.5mg) were precipitated in 80% (v/v) acetone at -20^{o}C, air dried and dissolved in 1.0ml Laemmli sample buffer (section 2.2.4 c). The sample was resolved into the constituent polypeptides of the complex on 10% (w/v) SDS-polyacrylamide preparative slab gels before staining with Coomassie blue (Fig. 4.2). After destaining, the gel was washed for 30

FIGURE 4.3 Reactivity of antiserum to bovine heart 2-oxoglutarate dehydrogenase complex with the individual subunits of the complex.

Various amounts of purified OGDC were subjected to SDS-PAGE. One half of the 10% (w/v) gel was stained with Coomassie blue (lanes 1-6); polypeptides on the other half of the gel were transferred to nitrocellulose paper and processed for immunodetection of antigenic polypeptides using (1:100 dil.) anti-OGDC serum (lanes 7-11).

Lane 1, M_r standards; lanes 2 & 7, 10µg purified OGDC; lanes 3 & 8, 5µg; lanes 4 & 9, 1µg; lanes 5 & 10, 0.5µg; lanes 6 & 11, 0.1µg.


M_r (x10^{-3})
92
67
45
30
E1
E3
E2
1 2 3 4 5 6
7 8 9 10 11

min in distilled water and protein bands, corresponding to the E1 and E2 polypeptides, were excised from the gel with a scalpel, diced, frozen in liquid nitrogen and ground finely using a mortar and pestle. The frozen powder was stored at -20°C until use.

For injection of the rabbit, 1g of powdered material, containing 100-300μg of protein, was homogenised with 0.5ml of distilled water in a glass tube using a tight-fitting teflon pestle. This suspension was mixed thoroughly with 1.0ml Freund's complete adjuvant and injected subcutaneously at multiple sites on the back and neck of a New Zealand White rabbit. Similar amounts were administered at 2-3 week intervals until, 10 days after the 4th set of injections, blood was drawn from a marginal ear vein. Additional booster injections were administered in Freund's incomplete adjuvant approx. two weeks before each bleeding. Serum was collected and stored in 1.0ml aliquots at -20°C.

4.3 RESULTS

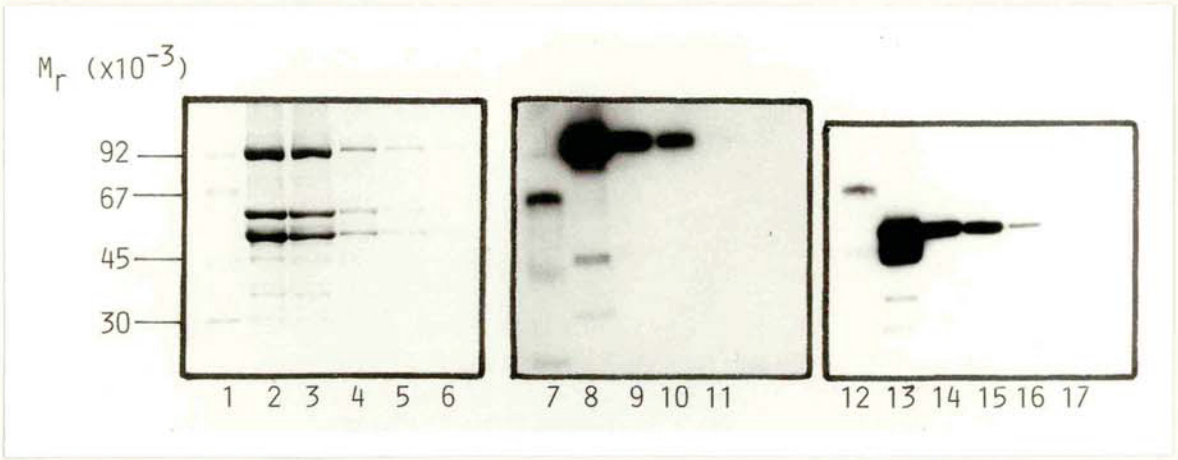
4.3.1 IMMUNOBLOTTING PATTERN OF PURIFIED 2-OXOGLUTARATE DEHYDROGENASE COMPLEX VERSUS ANTISERUM RAISED TO INTACT, NATIVE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

The reactivity of antiserum raised against intact, native OGDC (section 4.2.1) with the individual subunits of the complex was tested using immunoblotting analysis. Fig. 4.3 (lanes 2-5) shows the subunit composition when decreasing amounts of purified bovine heart OGDC were detected by Coomassie blue staining after polyacrylamide gel electrophoresis. Three major components were observed with M_r values 1035000 $\pm$ 1000 (E1), 54500 $\pm$ 1000 (E3) and 48500 $\pm$ 1000 (E2). However,

FIGURE 4.4 Reactivity of subunit specific antiserum with the individual subunits of the 2-oxoglutarate dehydrogenase complex.

Different amounts of OGDC were resolved into component enzymes by SDS-PAGE. One part of the 10% (w/v) slab gel was stained with Coomassie blue (lanes 1-6); polypeptides on the remainder of the gel were transferred to nitrocellulose and treated with (1:100 dil.) of anti-E1 serum (lanes 7-11) or anti-E2 serum (lanes 12-17).

Lanes 1,7 & 12, M_r standards; lanes 2-6, 10, 5, 1, 0.5 and 0.1μg respectively of purified OGDC. Lanes 8-11 and lanes 13-17, as lanes 3-6. Lane 17, 0.05μg.


M_r (x10^{-3})
92
67
45
30
1 2 3 4 5 6
7 8 9 10 11
12 13 14 15 16 17

when protein bands on a duplicate gel were electrophoretically transferred to nitrocellulose paper and probed with anti-OGDC serum to allow detection of antigenic polypeptides (see section 2.2.9 a) only two major bands were seen, corresponding in M_r to the E1 and E2 components of the complex. This technique is very sensitive and the antiserum was capable of detecting the E1 and E2 components of OGDC when as little as 0.5μg of purified OGDC was applied to the gel. However, no reaction with the E3 subunit was observed even with larger amounts (up to 10μg) of protein. The lower M_r bands observed at high levels of protein were probably proteolytic products resulting from degradation of OGDC subunits by endogenous proteases.

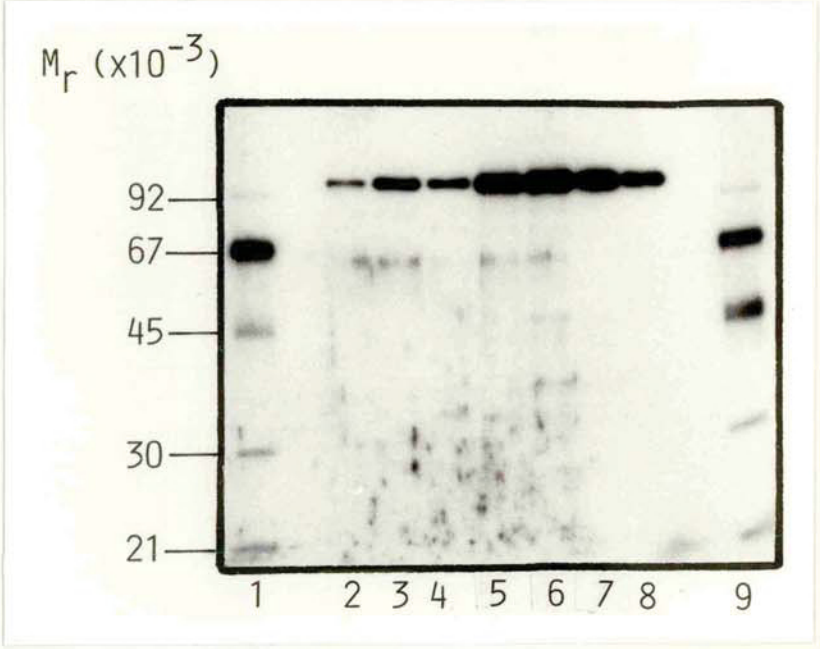
The poor antibody response elicited against E3 was also observed in antisera raised against intact, native bovine heart PDC (De Marcucci et al., 1985b) which has this subunit in common with OGDC. In both cases, OGDC and PDC, the titre of antibodies raised against E3 was <2% of that raised against the other subunits.

4.3.2 IMMUNOBLOTTING ANALYSIS OF ANTI-E1 AND ANTI-E2 SERA VERSUS PURIFIED 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

Immunoblotting analysis was again employed to check the specificity of the reaction of the subunit specific antisera, raised as described in section 4.2.2, with the individual components of OGDC. Fig. 4.4 shows the precise reactivities of anti-E1 and anti-E2 versus varying amounts of OGDC resolved on SDS-polyacrylamide gels. In each case, the subunit specific antiserum reacted only with the antigen to which it was raised indicating the absence of cross-contamination in the antigen. Some idea of the titre of the antisera was also gained.

FIGURE 4.5 Artefacts observed while using 2-mercaptoethanol in immune blotting analyses.

Protein fractions from different stages of the purification of OGDC from bovine heart were separated on a 10% (w/v) polyacrylamide gel. Following electrophoretic transfer of protein bands to nitrocellulose the replica was probed with anti-E1 serum (1:50 dil.). Immune complexes were located by incubation with ^{125}I-labelled protein A followed by autoradiography. Lanes 1 & 9, M_r standards; lanes 2-6, prepared in Laemmli sample buffer supplemented with 5% (v/v) 2-mercaptoethanol; lane 2, initial homogenate (20µg); lane 3, 1st extract (20µg); lane 4, 2nd extract (20µg); lane 5, 1st PEG precipitate, clarified (15µg); lane 6, 2nd PEG precipitate, clarified (15µg). Lanes 7 & 8, prepared in Laemmli sample buffer plus 10mM DTT; lane 7, material loaded onto Sepharose CL-2B (10µg); lane 8, purified OGDC (1µg).


M_r (x10^{-3})
92
67
45
30
21
1
2
3
4
5
6
7
8
9

It is seen that anti-E1 and anti-E2 sera were capable of detecting their parent antigens in 0.1μg and 0.05μg of purified OGDC respectively when employed under standard conditions at a dilution of 1:100 and on exposure of the X-ray film for 48h at -70°C. The additional lower M_r bands seen at high levels of protein loading are likely to be proteolytic fragments of the respective subunits.

At this time, it was decided not to attempt to raise antisera to E3. This decision was prompted by the comparatively poor antibody response elicited against E3 in comparison to the other subunits of OGDC. In addition, the method described in section 4.2.2 for the production of subunit specific antisera, involved multiple injections of relatively small amounts of protein and it was felt that this would not be a suitable regime for production of anti-E3 serum. It must also be stressed at this stage that the antisera to E1 and E2 were raised against the denatured polypeptide chains.

4.3.3 ARTEFACTS IN IMMUNOBLOTTING ANALYSES

In an attempt to demonstrate the absolute specificity of the antisera with respect to total cellular protein, anti-E1 serum was used to probe protein fractions from various stages in the purification of OGDC from bovine heart. The protein profile of these fractions has already been seen in Fig. 3.1. The corresponding immune replica in Fig. 4.5 reveals that anti-E1 serum is capable of detecting the E1 component of OGDC in these extracts with high specificity and sensitivity.

As can be seen from Fig. 4.5 (lanes 2-6), the immune replica of these samples, probed with anti-E1 serum, shows an additional band of M_r

approx. 65000 and a random distribution of dots over the autoradiograph. These artefacts were observed when the samples employed for SDS-PAGE had been prepared in Laemmli sample buffer supplemented with 5% (v/v) 2-mercaptoethanol. Both of these features are absent from lanes 7 & 8 which had been prepared in Laemmli sample buffer supplemented with 10mM DTT. It was demonstrated in two ways that the artefacts were caused by the presence of 2-mercaptoethanol and not the protein content of the samples (not shown). Firstly, an SDS-polyacrylamide gel was run with tracks containing Laemmli sample buffer in the absence of protein, Laemmli sample buffer plus 5% (v/v) 2-mercaptoethanol and Laemmli sample buffer supplemented with 10mM DTT. This gel was then treated as normal for immunoblotting analysis (section 2.2.9 a) and probed with anti-E1 serum. Only the tracks containing 2-mercaptoethanol contained the artefacts. Secondly, the samples run in lanes 2-6 of Fig. 4.5 were precipitated in 80% (v/v) acetone, air dried, then solubilised in Laemmli sample buffer plus 10mM DTT and subjected to immunoblotting analysis as before. The artefacts observed in Fig. 4.5 were not present in this analysis.

Therefore, in future experiments, all samples for immunoblotting analysis were prepared in Laemmli sample buffer supplemented with 10mM DTT.

4.3.4 EFFECT OF VARIOUS ANTISERA ON THE ACTIVITY OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

Once the specificity of the antisera with regard to the individual components of the complex had been established, their effects on overall OGDC activity were tested. It was found that

FIGURE 4.6 Effect of antiserum raised to intact, native 2-oxoglutarate dehydrogenase complex, subunit specific antisera and preimmune serum on the overall activity of the 2-oxoglutarate dehydrogenase complex.

Purified OGDC (approx. 20μg) was incubated with various amounts of antisera in a final volume of 200ul of 50mM sodium phosphate buffer, pH 7.0, containing 2.7mM EDTA, 1% (v/v) Triton X-100 and 0.15μM leupeptin for 90min at room temperature. Overall complex activity was measured before and after this incubation as described in section 2.2.1 a.

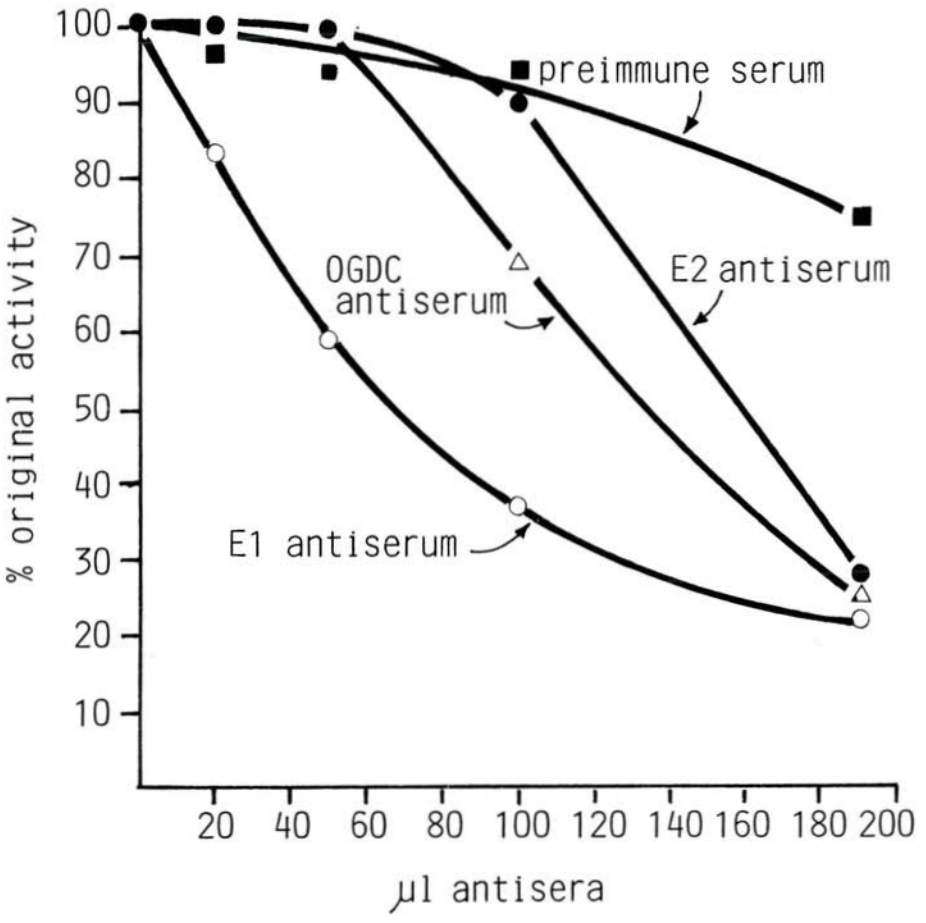

100
90
80
70
60
50
40
30
20
10
% original activity
preimmune serum
OGDC
antiserum
E2 antiserum
E1 antiserum
20
40
60
80
100
120
140
160
180
200
µl antisera

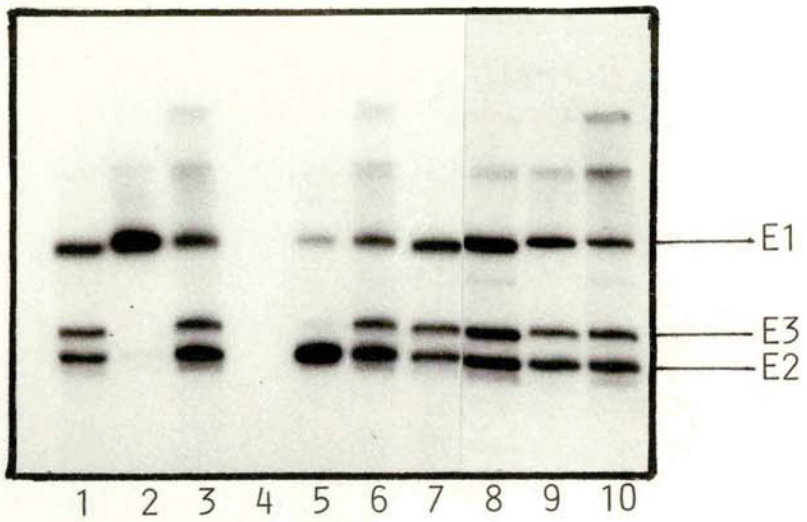
FIGURE 4.7 Immune precipitation of ^{3}H-labelled 2-oxoglutarate dehydrogenase complex from Triton buffer with anti-E1, anti-E2 sera and antiserum raised against the native complex.

OGDC, labelled with N-[^{3}H]ethylmaleimide, was incubated with antiserum raised against native OGDC or subunit specific antisera in Triton buffer (section 2.2.5 b). Immune complexes were adsorbed onto formalinised S. aureus cells before SDS-PAGE on 10% (w/v) gels followed by fluorography.

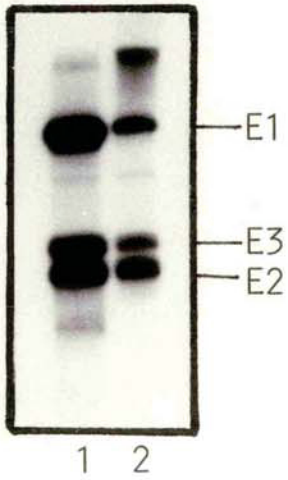
(A) Lanes 1, 7 & 10, ^{3}H-labelled OGDC; lane 2, anti-E1 serum; lane 3, anti-E1 serum, antigen heated to 100^{o}C for 2min before addition of antiserum; lane 4, preimmune serum; lanes 5 & 6 and lanes 8 & 9, as lanes 2 & 3 but with anti-E2 and anti-OGDC sera respectively.

(B) Lane 1, ^{3}H-labelled OGDC; lane 2, boiled antigen, no antiserum.

(A)



(B)



incubation of OGDC with antiserum raised against native, intact OGDC or with anti-E1 or anti-E2 subunit specific sera, resulted in inhibition of enzymic activity (Fig. 4.6). At the highest levels of antisera tested, OGDC activity was inhibited by approx. 75%. It is possible that complete inhibition of enzymic activity may occur at higher levels of antisera but whether this would be a true effect or due to an inhibitory factor in normal serum could not be determined. However, it is not unknown for antisera to only partially inhibit the enzymic activity of the antigen as the major antigenic determinants of the protein may be located such that the antibody only partially blocks access to the active site of the enzyme.

4.3.5 IMMUNE PRECIPITATION OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

To facilitate analysis of the components immunoprecipitated by the antisera raised against OGDC, the free thiol groups were labelled with N-[^{3}H]ethylmaleimide (section 2.2.5 b). The pattern of radiolabelled polypeptides (Fig. 4.7, lane 1) reveals that the three components of OGDC were labelled to approximately the same extent.

Different buffer systems and conditions of immune precipitation were tried in order to obtain optimal conditions for efficient, clean immune reactions and promote maximal dissociation of the complex. Two methods of precipitating the immune complexes were tried, namely Staphylococcus aureus cells or the double antibody technique. The former technique is based on the ability of protein A, a cell wall component of formalinised S. aureus cells, to bind the F_c portion of certain immunoglobulins in immune complexes, including rabbit IgG. The double antibody technique requires a second immune precipitation

employing an antiserum raised to the IgG of the initial species, in this case rabbit. This latter technique is more time consuming as it involves a second overnight precipitation. Moreover, it requires the addition of an optimal volume of second antibody which must be determined by titration. Addition of large volumes of second antibody also leads to problems in SDS-PAGE analyses of the products of immune precipitation. Heavy protein bands, corresponding to the individual chains of the IgG molecule, are seen on SDS-polyacrylamide gels causing distortion in these regions. The heavy chain of IgG causes distortion in the M_r region of 50000, which is one of the areas of interest in the study of OGDC whose E2 and E3 components have M_r values of 48500 and 54500 respectively. All immune precipitations were performed, therefore, using the S. aureus indirect immune precipitation technique. A commercial, standardised 10% (w/v) preparation of formalinised S. aureus cells, known as Pansorbin was employed in these studies.

Immune precipitations were carried out initially in 10mM Tris-HCl, pH 7.4, containing 300mM NaCl, 5mM EDTA and 1% (v/v) Triton X-100 (section 2.2.5 b). Since the complexes were dissociated by SDS during the labelling procedure, immune precipitation in this system should reflect the relative titres of antisera against the individual subunits of the complex. In the case of anti-OGDC serum, only the E1 and E2 subunits of the complex were precipitated by the antiserum (De Marcucci et al., 1985b) reflecting the relative titres already observed by immune blotting analysis (Fig. 4.2). It was also found that anti-PDC serum was capable of immune precipitating all components of ^3H-labelled PDC except E3 (De Marcucci et al., 1985b).

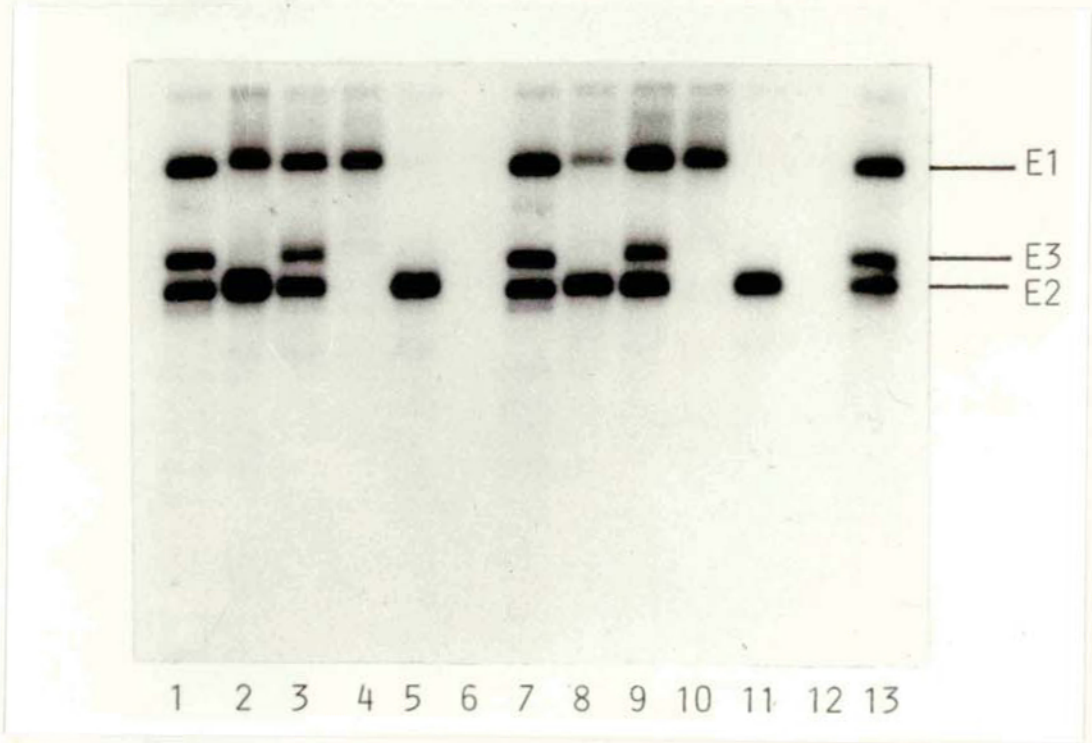
Fig. 4.7 (lane 8) illustrates that, although the titre of

antibody to E3 is low initially, in later bleeds of the same rabbit the titre has risen sufficiently to enable some immune precipitation of E3. In the case of the subunit specific antisera, anti-E1 serum immunoprecipitated the E1 component of the complex exclusively (Fig. 4.7, lane 2), indicating that the complex was completely dissociated. However, the precipitation carried out with anti-E2 serum contains some E1 in addition to E2. This was only occasionally observed and was probably due to incomplete dissociation and not cross reaction of anti-E2 serum with E1 which was ruled out by the earlier immunoblotting analysis (Fig. 4.4).

The eventual aim in these studies was to immune precipitate the component enzymes of OGDC from cellular extracts. Degradation of the polypeptides of interest by endogenous cellular proteases is one of the major problems of this procedure. One way of preventing proteolysis in such extracts is to boil the extract in the presence of detergent. Thus, the effect of boiling the antigen in Triton buffer before addition of antiserum was tested. It was also thought that heating the sample in this way might improve the degree of dissociation of the complex. Subsequent immune precipitation and analysis of the products revealed that, irrespective of the antisera used, all three component enzymes of OGDC were precipitated (Fig. 4.7, lanes 3,6 & 9). Further experiments in this system showed that even in the absence of antisera the final product of precipitation contained the three subunits of OGDC (Fig. 4.7B). Either the complex had formed aggregates large enough to spin down independently during centrifugation of formalinised S. aureus cells or heating had denatured the complex components such that they adsorbed, non-

FIGURE 4.8 Immune precipitation of ^{3}H-labelled 2-oxoglutarate dehydrogenase complex from 3D-TKM buffer with antisera raised against intact 2-oxogluarate dehydrogenase complex, E1 and E2.

OGDC, labelled with N-[^{3}H]ethylmaleimide, was dissociated in 3D-TKM buffer (section 2.2.8 b) and immune precipitated with antisera raised to native OGDC and subunit specific antisera. Lanes 1,7 & 13, ^{3}H-labelled OGDC. Immune precipitates of ^{3}H-labelled OGDC with native OGDC antiserum, bleed 1 (lane 2), bleed 6, (lane 3); E1 subunit specific antiserum (lane 4); anti-E2 serum (lane 5) and preimmune serum (lane 6). Lanes 8-12, as lanes 2-6 but with antigen boiled before addition of antiserum.

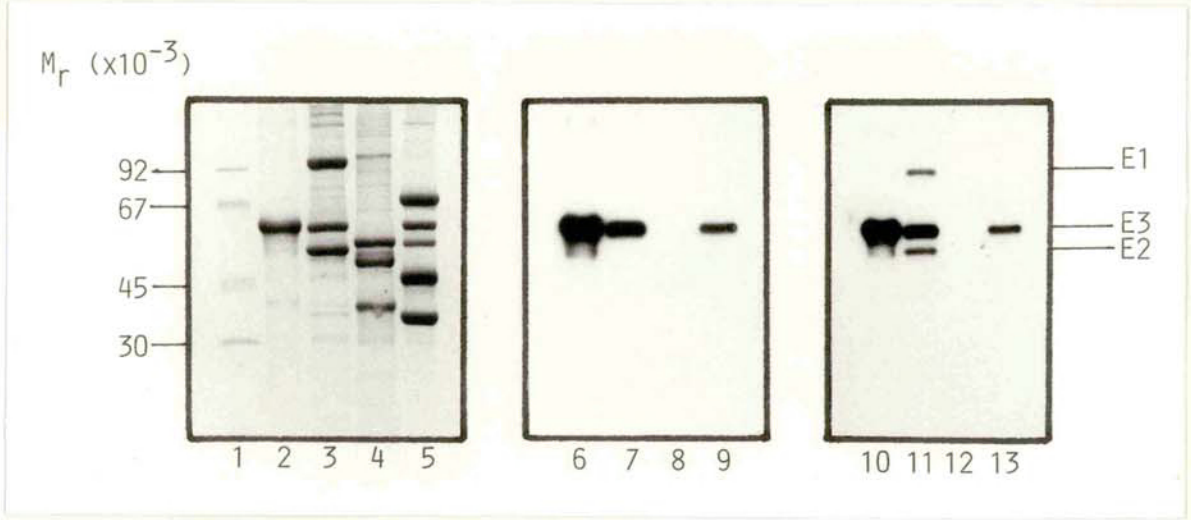

E1
E3
E2
1 2 3 4 5 6 7 8 9 10 11 12 13

specifically, to the S. aureus cells and were only dislodged by further boiling in Laemmli sample buffer prior to SDS-polyacrylamide gel analysis.

Immune precipitation in the 3D-TKM (section 2.2.8 b) buffer system is seen in Fig. 4.8. This buffer provides a much more stringent set of conditions for immune precipitation as 0.5% (w/v) SDS and 1% (w/v) DOC are present in the buffer in addition to 1% (v/v) Triton X-100. Accordingly, complete dissociation is observed and each of the subunit specific antisera immunoprecipitate only their parent antigen. Again the E3 component is absent from the products of immune precipitation carried out with anti-OGDC serum from the initial bleeds of the rabbit. However, it is present in immune precipitations employing serum from later bleeds of the same rabbit, indicating a rise in antibody titre to E3. In contrast to the immune precipitations carried out in Triton buffer, boiling the antigen in 3D-TKM before addition of antisera, had little or no effect on the products of immune precipitation (Fig. 4.8, lanes 8-12). One possible explanation for this, based on the previous theory of denatured polypeptides binding non-specifically to Pansorbin, is that although the denatured subunits adsorb to the S. aureus cells, as may be the case in Triton buffer, the washing conditions in this case, which include the detergents, SDS and DOC, were stringent enough to remove these contaminant bands while at the same time being mild enough to allow the association of antigen and antibody to proceed. It is also possible that, if boiling in Triton buffer resulted in the formation of large aggregates of OGDC, the presence of the extra detergents in 3D-TKM would prevent this aggregation occurring when the antigen was heated to 100^{o}C.

FIGURE 4.9 Reactivity of anti-E3 serum and antiserum raised to 2-oxoglutarate dehydrogenase complex with purified E3 and the three mitochondrial 2-oxo acid dehydrogenase complexes.

Purified E3 from porcine heart, OGDC and PDC from bovine heart and BCDC from bovine kidney were resolved on a 10% (w/v) SDS-polyacrylamide gel slab. One set of tracks was stained with Coomassie blue (lanes 1-5); lane 1, M_r standards; lane 2, E3 (5μg); lane 3, OGDC (10μg); lane 4, BCDC (10μg); lane 5, PDC (15μg). The protein in the duplicate sets of tracks was transferred to nitrocellulose and processed for immunological detection of E3 with 1:50 dil. of anti-E3 serum (lanes 6-9 as lanes 2-5, 2ug per track). The nitrocellulose was then overlayed with anti-OGDC serum (1:50 dil.) and the dried paper re-exposed to X-ray film (lanes 10-13 as lanes 6-9).


M_r (x10^{-3})
92
67
45
30
1 2 3 4 5
6 7 8 9
10 11 12 13
E1
E3
E2

4.3.6 PREPARATION AND CHARACTERISATION OF ANTI-E3 SERUM

Antiserum was raised against purified E3 by Miss Anne Phelps in our laboratory. Samples of a commercially purified porcine heart lipoamide dehydrogenase (1mg) were employed to immunise a rabbit following a regime similar to that used to raise antiserum to intact, native OGDC (section 4.2.1). The rabbit received four injections of E3 (1mg) in Freund's complete adjuvant before the initial bleeding. Subsequent booster injections of E3 (1mg) were carried out in Freund's incomplete adjuvant. Serum collected was again stored at $-20^{\circ}C$ in aliquots of 1.0ml.

Immunoblotting analysis was employed to determine the cross reaction of this antiserum with purified E3 and the E3 component of the three 2-oxo acid dehydrogenase complexes. Samples of purified porcine heart E3, bovine heart OGDC, bovine kidney BCDC (a gift from G.H.D. Clarkson) and bovine heart PDC (a gift from O.L. De Marcucci) were resolved on a 10% (w/v) polyacrylamide gel and visualised by staining in Coomassie blue (Fig. 4.9, lanes 2-5 respectively). Bovine heart PDC is composed of 5 protein bands of M_r 70000 (E2), 55000 (E3), 50000 (component X), 42000 (E1α) and 37000 (E1β) (Linn *et al.*, 1972; Stanley & Perham, 1980; De Marcucci & Lindsay, 1985) whereas bovine kidney BCDC is resolved into 3 polypeptides of M_r 52000 (E2), 46000 (E1α) and 35000 (E1β). The E3 component is missing from BCDC purified by this procedure (Lawson *et al.*, 1983). The absence of E3 from the sample of BCDC is confirmed by examination of the immune replica of these samples probed with anti-E3 serum (Fig. 4.9, lane 8). This antiserum is seen to react specifically with the purified porcine

FIGURE 4.10 Immune precipitation of ^{3}H-labelled 2-oxoglutarate dehydrogenase complex with anti-E3 serum and antisera raised against native 2-oxogluarate dehydrogenase and pyruvate dehydrogenase complexes.

Pure ^{3}H-labelled OGDC was immune precipitated from 3D-TKM buffer using anti-E3, anti-PDC or anti-OGDC sera. Products of immune precipitation were separated by SDS-PAGE on 10% (w/v) gels and visualised using fluorography.
Lane 1, ^{3}H-labelled OGDC; lane 2, anti-E3 serum; lanes 3 & 4, anti-OGDC serum; lane 5, anti PDC serum. Tracks 1-3 were exposed to X-ray film for 5 days and tracks 4 & 5 for 10 days.

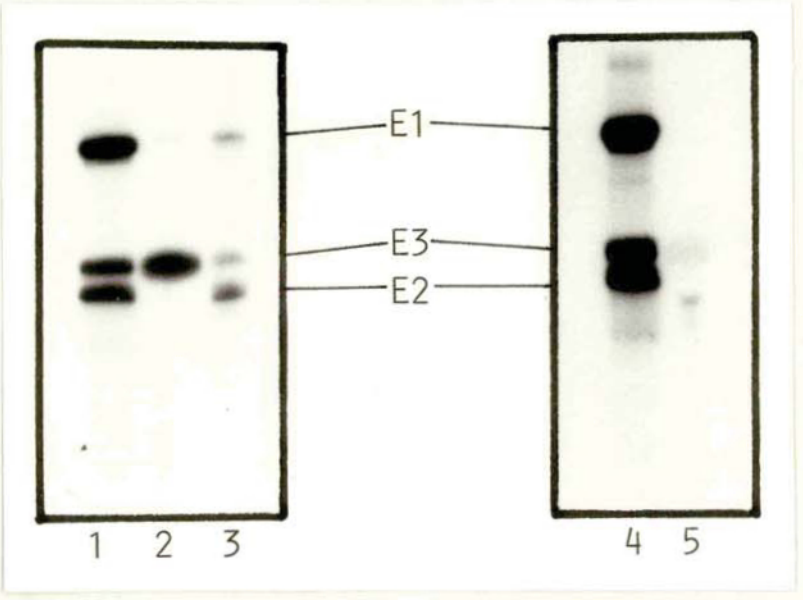
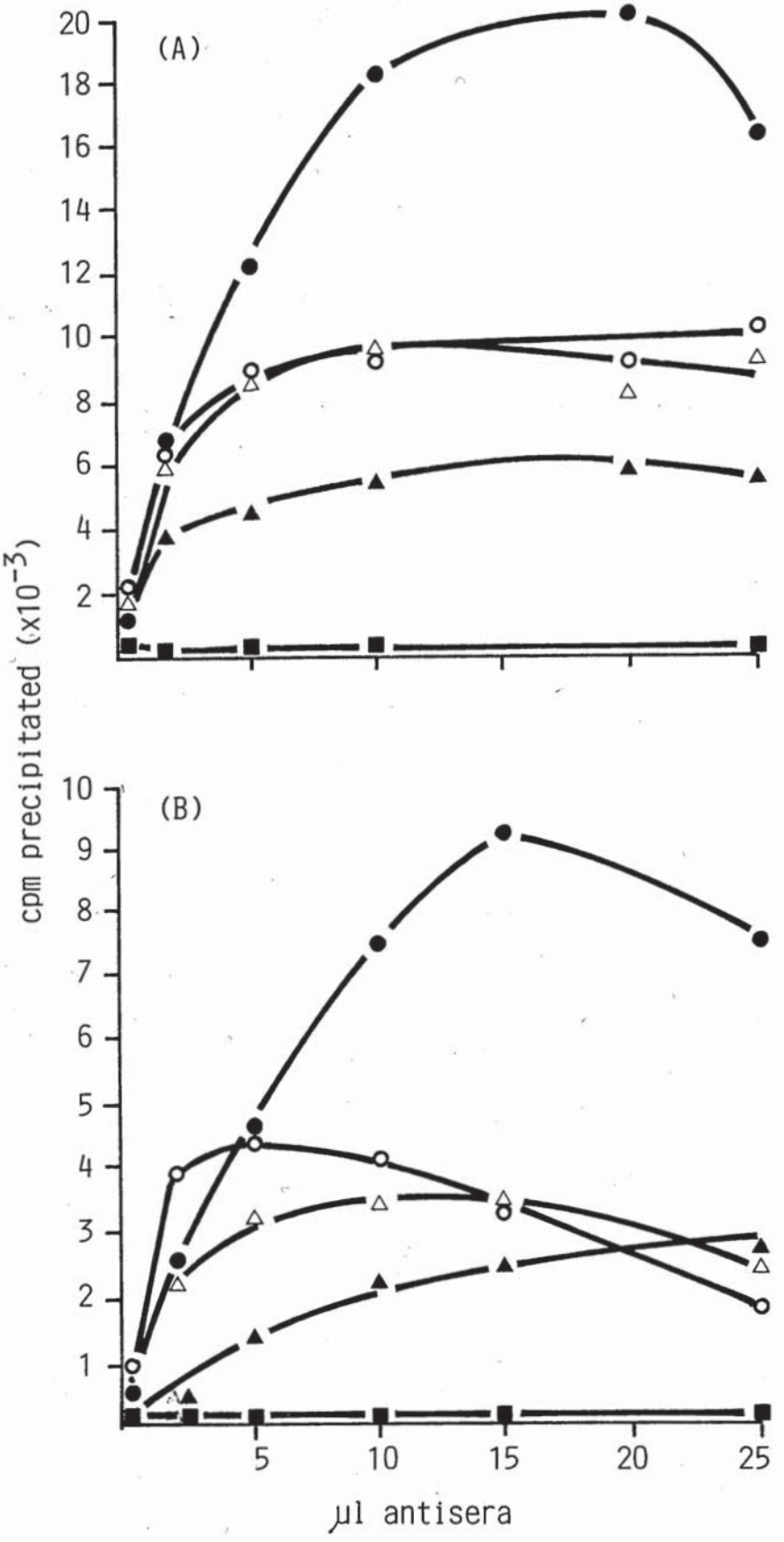

E1
E3
E2
1
2
3
4
5

FIGURE 4.11 Titration of ^{3}H-labelled 2-oxoglutarate dehydrogenase complex with varying amounts of antisera raised to 2-oxoglutarate dehydrogenase complex and its component enzymes.

Pure ^{3}H-labelled OGDC (0.2µg: approx 30000c.p.m.) was immune precipitated from 100µl of either Triton buffer(A) or 3D-TKM(B) with varying amounts of anti-OGDC serum or subunit specific antisera. Immune complexes were adsorbed onto formalinised S. aureus cells, collected by centrifugation and washed as described in section 2.2.9 b). Final pellets were dissolved in 50µl of Laemmli sample buffer and 10µl of this was subjected to scintillation counting.

- ● anti-OGDC serum, bleed 6
- ⊙ E1 subunit specific antiserum
- Δ anti-E2 serum
- ▲ anti-E3 serum
- ■ preimmune serum


(A)
(B)
cpm precipitated (x10⁻³)
µl antisera
20
18
16
14
12
10
8
6
4
2
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25

heart E3 and the E3 components of both OGDC and PDC from bovine heart. After exposure of the X-ray film to yield the image in lanes 6-9 of Fig. 4.9, the nitrocellulose paper was then overlayed with anti-OGDC serum. The only additional reaction seen was with the E1 and E2 components of the OGDC complex (Fig. 4.9, lanes 10-13). Antiserum raised against OGDC did not show cross reaction with the E1 or E2 components of either PDC or BCDC indicating that unlike the E3 components there is no immunological similarity between the E1 and E2 subunits of the different 2-oxo acid dehydrogenase complexes. The ability of E3 subunit specific antisera to immunoprecipitate the E3 component of ^{3}H-labelled OGDC is revealed in Fig. 4.10. It was also seen that antiserum raised against bovine heart PDC had the capacity to immunoprecipitate small amounts of the E3 component of ^{3}H-labelled OGDC. This would be expected to occur if lipoamide dehydrogenase (E3) was indeed common to the two multi-enzyme assemblies. The low yield of E3 in the immune precipitates with PDC antiserum is probably related to the low titre of antibodies to E3 in this serum.

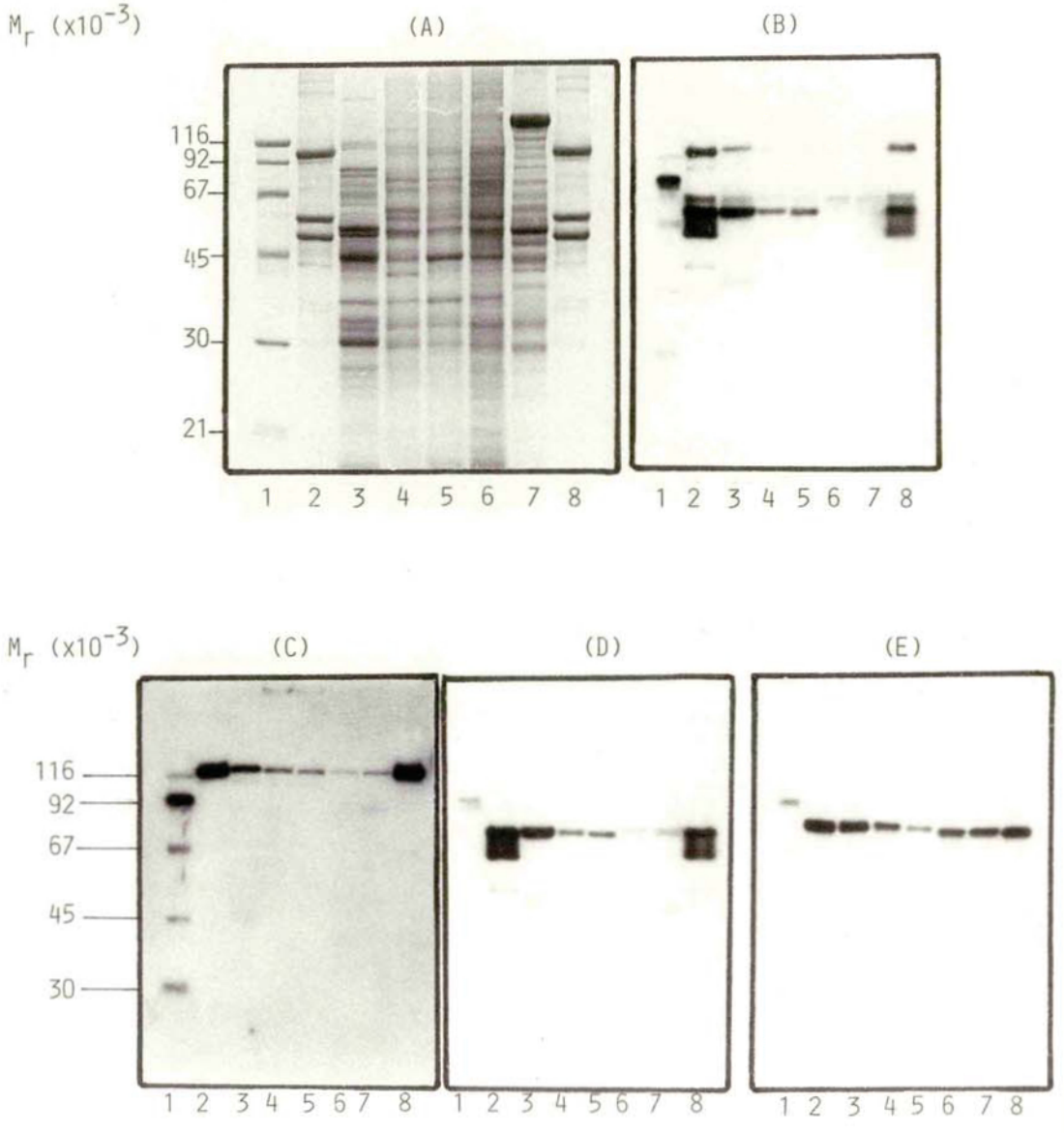
4.3.7 TITRATION OF ^{3}H-LABELLED 2-OXOGLUTARATE DEHYDROGENASE COMPLEX WITH ANTISERUM TO NATIVE, INTACT 2-OXOGLUTARATE DEHYDROGENASE COMPLEX AND SUBUNIT SPECIFIC ANTISERA

A constant amount of ^{3}H-labelled OGDC was incubated with various volumes of anti-OGDC serum or subunit specific antisera, and the extent of immune precipitation (c.p.m.) was determined in each case. Fig. 4.11A shows that, in the presence of Triton buffer, approximately 66% of total c.p.m. can by precipitated with anti-OGDC serum whereas the subunit specific antisera allows precipitation of 20-35% of the

FIGURE 4.12 Cross reactivity of antisera with mitochondria from various sources.

Mitochondria purified from various sources were separated by SDS-PAGE on 10% (w/v) polyacrylamide slab gels. One gel was stained with Coomassie blue to visualise the protein bands (A). Protein bands on the duplicate gels were electrophoretically transferred to nitrocellulose and processed for detection of antigenic polypeptides using native OGDC antiserum, bleed 6 (B); anti-E1 serum (C); E2 subunit specific antiserum (D) and anti-E3 serum (E).

Lane 1, M_r standards; lanes 2 & 8, pure OGDC (10μg in A; 2μg in B-E); lane 3, bovine heart mitochondria (50μg); lanes 4-6, mitochondria from PK-15, NBL-1 and BRL cell lines respectively (50μg); lane 7, rat liver mitochondria (50μg).


M_r (x10^{-3})
(A)
(B)
116
92
67
45
30
21
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
M_r (x10^{-3})
(C)
(D)
(E)
116
92
67
45
30
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

total OGDC. The supernatant fluid was not analysed following immune precipitation and the remaining radioactivity could represent soluble counts. The difference in radioactivity precipitated using antiserum to OGDC as opposed to subunit specific antisera probably reflects the number of radiolabelled protein bands in the immune complexes. In the case of OGDC antiserum, three radiolabelled protein bands are precipitated whereas with subunit specific antisera only one radioactive band is present in the immune complex.

In comparison the percentage of total c.p.m. precipitated in the presence of 3D-TKM buffer (Fig. 4.11B) is approximately 50% of that achieved in the presence of Triton buffer. This probably reflects the increased stringency of the washing conditions where only high affinity binding is preserved.

4.3.8 CHOICE OF CELL LINE IN WHICH TO CARRY OUT FURTHER STUDIES

Three continuous cell lines were available in which to carry out biosynthetic studies: NBL-1, a bovine kidney cell line; PK-15, porcine kidney cells; and BRL, buffalo rat liver cells. Immune blotting analysis of these cell lines was carried out to determine the most suitable culture in which to pursue the biosynthetic studies.

Total mitochondrial protein from various cell types, purified bovine heart OGDC and M_r standards were separated electrophoretically on 10% (w/v) SDS-polyacrylamide slab gels. The pattern of staining with Coomassie blue is illustrated in Fig. 4.12A while Fig. 4.12B-E shows the corresponding immunoblots with anti-OGDC, anti-E1, anti-E2 and anti-E3 sera respectively. From these data, it is apparent that the antisera is highly specific for the constituent polypeptides of the

OGDC complex, reacting exclusively with the E1, E2 and E3 subunits in crude mitochondrial extracts. The extra band seen in Fig. 4.12B & D in the lanes corresponding to purified OGDC represents the major proteolytic fragment of E2, seen when the purified OGDC is subjected to repeated freezing and thawing. In the majority of cases (except anti-E3 serum), the antisera show strong cross reaction with bovine heart mitochondria (lane 3) and mitochondria prepared from porcine kidney (lane 4) and bovine kidney cells (lane 5). In comparison, a weaker cross reaction is exhibited against mitochondrial extracts from BRL cells or rat liver. With anti-E3 serum, a strong cross reaction is seen with all extracts.

4.4 DISCUSSION

A major observation of this work is that the lipoamide dehydrogenase (E3) component of OGDC elicits a poor antibody response relative to the other component enzymes of the complex. This low immunogenicity of the common E3 subunit is also apparent in antisera raised to PDC (De Marcucci *et al.*, 1985b).

Evidence supporting this conclusion was obtained by (a) employing immunoblotting to examine the precise reactivity of anti-OGDC serum versus the purified enzyme and (b) indirect immune precipitation of dissociated ^{3}H-labelled OGDC. In both cases, a lack of reaction of antiserum with lipoamide dehydrogenase is observed. In the latter case, E3 is found in the supernatant fraction after immune precipitation (not shown) ruling out the possibility of selective degradation of this component on incubation with antisera or with the formalinised *S. aureus* cells.

This apparent low immunogenicity cannot be attributed to a low content of E3 in these purified multienzyme complexes as the polypeptide chain ratios of E1, E2 and E3 are 1:2:1 and 5:5:1 in OGDC and PDC respectively (De Marcucci et al., 1985b). Also since the E3 component is thought to be present on the surface of both multienzyme assemblies (Oliver & Reed, 1982), inaccessibility of the antigen cannot be the cause of the lack of antibody response.

One plausible explanation for these data is that the low immunogenicity of E3 reflects a high degree of conservation of amino acid sequence in this polypeptide chain. This seems feasible in view of the fact that E3 is a common component of three multienzyme assemblies and therefore severe constraints must be imposed on amino acid substitution by the multiple and differing subunit specific interactions involved in the three structurally and functionally analogous 2-oxo acid dehydrogenase complexes. Indeed, the complete sequence of E. coli E3 has been deduced from examination of the cloned gene (Stephens et al., 1983) and considerable homologies are detected between the sequence of the E. coli enzyme and tryptic peptides of the porcine heart E3 (Stephens et al., 1983; Arscott et al., 1984).

The ability to raise subunit specific antiserum directed against purified E3 is probably related to the large amounts of protein employed in the immunisation protocol. Immunoblotting analysis using this antiserum, revealed an absence of the E3 component from purified bovine kidney BCDC.

Although it is generally accepted that E3 is common to all three 2-oxo acid dehydrogenase complexes, this has been shown to be false in the case of Pseudomonas putida (Sokatch et al., 1981a). A lipoamide dehydrogenase component of M_r 56000 was purified from P. putida cells

grown in the presence of glucose, which contain OGDC and PDC activities. However, E3 from cells grown plus valine, which contain all three 2-oxo acid dehydrogenase complex activities, has an M_r of 49000 and differs from the E3 purified from glucose grown cells in pH optimum also. It was concluded that the latter E3, of M_r 49000, was specific for BCDC and the former for the other two 2-oxo acid dehydrogenase complexes.

Multiple forms of mammalian E3 have been observed, although it is thought that these are all different conformations of the same protein (Sakurai *et al.*, 1970). The assumption that there is only one mammalian E3, common to all three 2-oxo acid dehydrogenase complexes, can only be proved by the demonstration of only one gene for this particular protein. This has been demonstrated in *E. coli* where E3 is common to OGDC and PDC (Guest & Creaghan, 1973; Guest, 1974).

The high selectivity and sensitivity of the antisera were monitored by immunoblotting against whole cell and mitochondrial extracts. Transfer of proteins to nitrocellulose paper has several advantages: filters are easier to manage, proteins are more accessible and therefore processing times and amounts of reagent required are reduced, the same replica can be used for successive analyses. One of the most commonly encountered artefacts of this technique is that elution is dependent on M_r. However, addition of 0.02% (w/v) SDS to the original transfer buffer of Towbin *et al.* (1979) facilitates the elution of higher M_r proteins. After transfer of the protein to nitrocellulose, unbound sites on the paper must be quenched prior to treatment with antiserum. Initially this was achieved with 3% (w/v) BSA in the washing buffer as described by Towbin *et al.* (1979).

However, darkened backgrounds were observed so the non-ionic detergent Tween 20 was employed as a blocking agent (Batteiger et al., 1982). Improved backgrounds and specificity of interaction were achieved by this procedure. There was the additional advantage that the replicas could be stained for protein after use to check the efficiency and fidelity of transfer.

The observation that artefactual bands appear on SDS-polyacrylamide gels when 2-mercaptoethanol is present in the sample buffer has been noted previously (Tasheva & Dessev, 1983). These authors claimed that 2-mercaptoethanol was responsible for the appearance of two non protein bands (M_r 54000 and 65000) in Coomassie blue or silver-stained gels. It was reported that this effect was observed on electrophoresis of pure mercaptoethanol but, in contrast to observations in this work, it was also suggested that this effect became even more pronounced when 2-mercaptoethanol was replaced by DTT.

The highly sensitive immunoblotting technique, using samples prepared in Laemmli sample buffer plus 10mM DTT, revealed the specificity of the antisera which react exclusively with the respective antigens to which they were raised. It also revealed the fact that antisera raised to bovine heart OGDC can cross react with the corresponding polypeptides of OGDC in porcine kidney, bovine kidney and rat liver cells. Similarly antiserum raised to porcine heart E3 cross reacts with lipoamide dehydrogenase from the afore-mentioned cell lines and also with bovine heart E3. It was not established whether the weaker cross reaction observed against cells derived from rat liver tissues reflected a genuine immunological difference or merely a lower abundance of the multienzyme complex in these tissues.

However, on the basis of these results, biosynthetic analyses were initiated in both porcine kidney (PK-15) and bovine kidney (NBL-1) cells.

CHAPTER FIVE

IMMUNE PRECIPITATION OF THE COMPONENT ENZYMES OF 2-OXOGLUTARATE DEHYDROGENASE FROM MAMMALIAN CELLS

5.1 INTRODUCTION

In the previous chapter the preparation of antisera to native, whole OGDC, native E3 and the denatured E1 and E2 subunits was described. The high specificity and sensitivity of these antisera was determined by immune blotting analysis versus purified bovine heart OGDC, porcine heart E3 and crude cellular or mitochondrial extracts. In addition, the ability of these antisera to immune precipitate the component enzymes of OGDC was demonstrated using OGDC labelled with N-[^{3}H]ethylmaleimide. The next stage in the project involved immune precipitation of the subunits of OGDC from crude extracts of [^{35}S]methionine-labelled cell cultures.

Immunoblotting analysis revealed that porcine kidney (PK-15) or bovine kidney (NBL-1) were the best cell lines in which to carry out biosynthetic studies. These cell lines were chosen initially because they produced very little lactic acid, suggesting a high degree of aerobic metabolism and hopefully a high content of mitochondria. It was also thought that, since they were closely related to bovine heart, cross reaction with the antisera would occur. As the immunoblotting studies showed this assumption proved valid and the antisera cross reacted strongly with the component enzymes of OGDC in both PK-15 and NBL-1 cell lines. This chapter deals with determining optimal conditions for labelling the cells with [^{35}S]methionine and reproducible immunoprecipitation of the subunits of OGDC from detergent extracts of these [^{35}S]methionine-labelled cells. It also concerns the detection of precursor forms of the component enzymes of OGDC in cells labelled with [^{35}S]methionine in the presence of uncouplers of oxidative phosphorylation.

5.2 OPTIMISATION OF CONDITIONS FOR LABELLING CELLS

Considerable effort was expended in determining optimal conditions for incorporation of [^{35}S]methionine into cellular protein. The effect of various media (NGM, LMM, MMM: section 2.2.7) and the presence or absence of serum were tested for their effect on incorporation of [^{35}S]methionine into protein. Cells were introduced into 5cm diam. petri-dishes in 3ml NGM at a concentration of 250000 cells/ml. Petri-dishes were incubated at 37^{o}C in a humidified incubator for 24-48h until semi-confluent monolayers were observed. NGM was replaced by 2ml of one of the above media, made up with or without serum (in those media made up without serum the volume was kept constant by addition of sterile distilled water), after an initial rinse in the same medium. 10μCi of [^{35}S]methionine was added to the dishes which were incubated at 37^{o}C for 30 min. After this time radioactive medium was removed and the cell monolayers washed three times in ice-cold PBS before the cells were harvested in 0.5ml of Triton buffer (section 2.2.5 b). 50μl aliquots were spotted onto Whatman filter discs, which were processed as described in section 2.2.6 b, to determine [^{35}S]methionine incorporation into protein. In the presence of NGM, incorporation of radioactivity into protein was 20-25% of that incorporated in either LMM or MMM. The presence or absence of serum had very little effect on incorporation. Highest levels of [^{35}S]methionine in protein were observed in cells incubated in MMM. Incorporation in LMM was approx. 70% of that in MMM. However, comparison of methionine levels in the various media revealed that the concentration in LMM is 5μM whereas that in MMM is 0.7μM. Therefore, it

was expected that incorporation in MMM would be much greater than that in LMM. As this was not the case,it suggests that either there was an intracellular pool of methionine large enough to make this difference negligible or that the lack of methionine had an adverse effect on protein synthesis and cell growth. In order to avoid this possible adverse effect on protein synthesis, all further labelling was carried out in LMM. Serum was also included in the labelling medium since removal of serum did not significantly increase the degree of incorporation. In the presence of serum normal growth and cell division is more likely to take place.

The effect of preincubation in LMM, prior to addition of radioactive methionine, was tested also. Preincubation in LMM was seen to increase incorporation of [^{35}S]methionine into protein with an optimal incubation time of 1h in LMM, prior to addition of the radioactive amino acid. This may indicate the presence of an intracellular pool of methionine which is depleted after an incubation period of 1h.

The time course of incorporation of [^{35}S]methionine into protein over 24h was investigated. Cells were introduced into 2.5cm diam. petri-dishes at a concentration of 300000 cells/ml in 1ml of NGM. Petri-dishes were incubated at 37^{o}C until semi-confluent monolayers were observed. Monolayers were rinsed in LMM and incubated in 1ml of LMM for 1h at 37^{o}C prior to the addition of 5μCi [^{35}S]methionine/dish. The incorporation of radioactivity into protein after various times was determined as described earlier. It was found that incorporation of [^{35}S]methionine was linear for 8h after which the majority of the radioactivity had been incorporated.

The concentration of cells introduced initially into petri-dishes was determined by trial and error. As already mentioned the levels used were 250000-350000 cells/ml and the final volume depended on the size of the petri-dish. The amount of [^{35}S]methionine added was a compromise between c.p.m. incorporated over the period of interest and cost limitations.

In general, cells were labelled for 4h or overnight with 100-200μCi [^{35}S]methionine in LMM after a 1h preincubation in the same medium.

5.3 IMMUNE PRECIPITATION OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX FROM PORCINE KIDNEY CELLS

Initial attempts to immunoprecipitate the component enzymes of OGDC from crude extracts of cultured cells involved preparing cell extracts by homogenisation in Triton buffer. The products of immune precipitation from these extracts contained the subunits of OGDC but were also heavily contaminated with other cellular proteins. In the preparation of cellular extracts by this method, the nuclei were lysed with concomitant release of DNA. The presence of this 'sticky' genetic material was probably one of the major causes of this contamination. Preincubation of the extracts with Pansorbin for 1h at room temperature, followed by removal of the Pansorbin by centrifugation at 14000g for 2 min and subsequent immune precipitation of the supernatant fluid, was tried in an attempt to eliminate the contamination. No improvement was observed.

The double antibody indirect immune precipitation technique was also employed. Results were similar to those obtained using Pansorbin

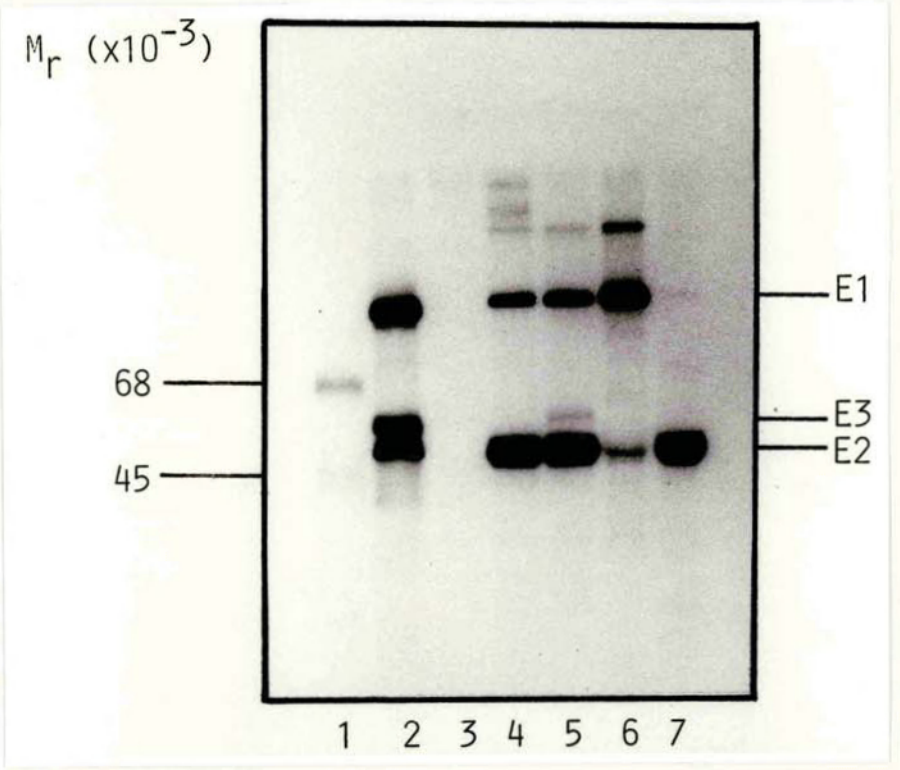
and again there were problems of distortion in the gels (section 4.3.5). Pre-adsorption of the extracts with pre-immune serum and removal of the immune complexes using goat anti-rabbit IgG prior to immune precipitation was attempted with no resultant decrease in the degree of contamination.

Attempts to improve the quality of the immune complex-Pansorbin assembly were also made by passage through a sucrose gradient. Products of immune precipitation, after three washes in buffer plus detergent (section 2.2.9 c) were resuspended in 50µl of buffer plus detergent. Sucrose gradients of 300µl each of 0.5M and 1M sucrose in detergent containing buffer were prepared. The immune products were layered on top of these gradients and centrifuged at 3000g for 30 min at room temperature. Sucrose was removed with a Pasteur pipette and the pellets taken up in Laemmli sample buffer and analysed by SDS-PAGE (section 2.2.4). Again no improvement in the degree of contamination was observed.

A procedure for preparing extracts such that the nuclei remained intact had been developed previously for studies on the biosynthesis of immunoglobulins (Mosmann et al., 1979). Cells were harvested in buffer containing 1% (v/v) Triton X-100, lysing the cells but not the nuclei which were then removed by centrifugation. The detergent content of the buffer was adjusted to equal that of 3D-TKM (section 2.2.8 b) and the mixture clarified by centrifugation before addition of the antisera. This buffer gave minimal non-specific adsorption of contaminating material in control immune precipitates while also promoting maximal dissociation of the multienzyme complex. Pre-adsorption with Pansorbin was found to be unnecessary as was sucrose gradient centrifugation of the immune complexes.

FIGURE 5.1 Immune precipitation of 2-oxoglutarate dehydrogenase complex from porcine kidney cells.

[^{35}S]methionine-labelled cell extract from PK-15 cells, prepared as described in section 2.2.8 a, was incubated with various antisera. Immune complexes were precipitated using formalinised *S. aureus* cells and then analysed by SDS-PAGE on 10% (w/v) slab gels followed by fluorography.
Lane 1, M_r standards; lane 2, ^{3}H-labelled OGDC. Immune precipitates of PK-15 cell extracts using preimmune serum (lane 3); native OGDC antiserum, bleed 1 (lane 4) and bleed 6 (lane 5); anti-E1 serum (lane 6) and anti-E2 serum (lane 7).


M_r (x10^{-3})
68
45
E1
E3
E2
1 2 3 4 5 6 7

Immune precipitation profiles, observed after the various antisera were employed to select specifically OGDC or individual subunits from [^{35}S]methionine-labelled detergent extracts of porcine kidney cells grown in culture, are seen in Fig. 5.1. Even under these stringent conditions, on occasions, incomplete dissociation could be detected. For example, anti-E1 immune precipitate (lane 5) contains a small amount of associated E2.

Under these conditions, the pattern of immune precipitation again reflects the titre of antibodies raised against the individual subunits. It is clear that antiserum to the native complex is initially capable of inducing specific precipitation of the E1 and E2 subunits only. The exclusion of E3 from the final immune complex indicates that the titre of antibody to this component is low in comparison to the other major enzymes. However, as already observed (section 4.3.5), in later bleeds of the same rabbit it is apparent that the antibody titre has increased substantially to the extent that some precipitation of this component can be achieved also.

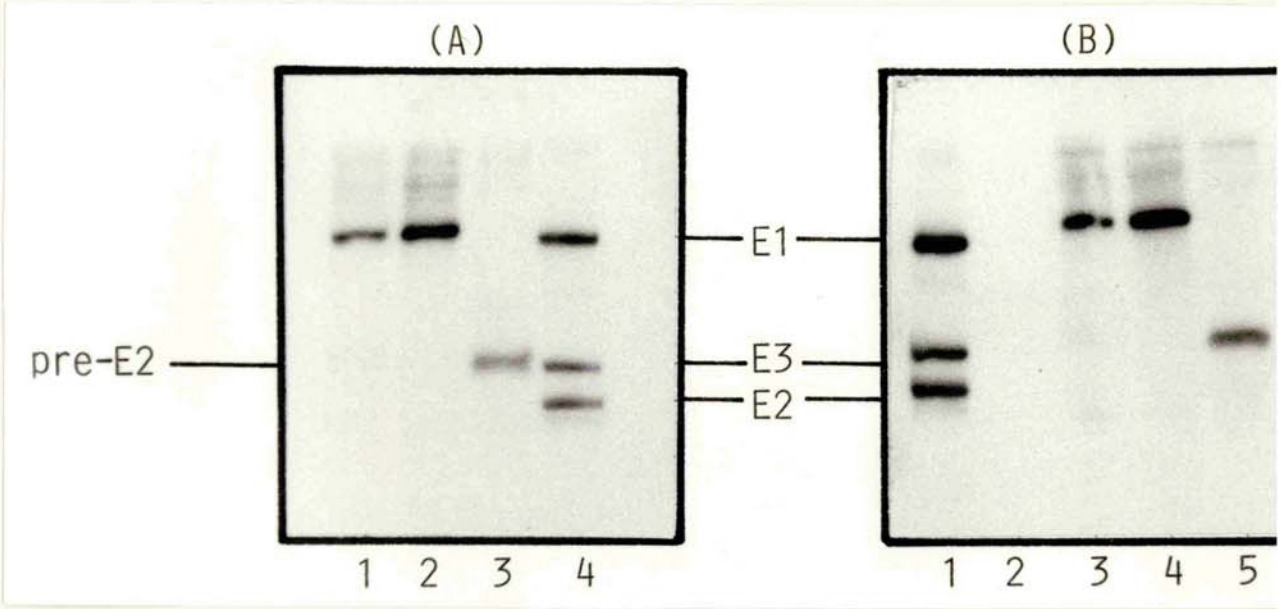
These results establish (a) the monospecific nature of the immune reaction in [^{35}S]methionine-labelled crude cell extracts and (b) that antisera to the bovine heart enzymes can induce strong and efficient immune precipitation of the equivalent complex from porcine kidney cells. Similar highly specific and efficient immune precipitation of the component enzymes of OGDC was observed from [^{35}S]methionine-labelled detergent extracts of bovine kidney cells (not shown).

FIGURE 5.2 Detection of 2-oxoglutarate dehydrogenase complex subunits in porcine kidney cells labelled with [^{35}S]methionine in the presence of uncouplers of oxidative phosphorylation.

Extracts were prepared from cells labelled with [^{35}S]methionine for 4h in the presence of 10μM FCCP (A) or 2mM 2,4-DNP (B). Immune precipitation was carried out using anti-OGDC serum or subunit specific antisera directed against E1 or E2. The products of immune precipitation were analysed on 10% (w/v) SDS-polyacrlamide gels.

(A) Lane 1, anti-OGDC serum; lane 2, anti-E1 serum; lane 3, anti-E2 serum; lane 4, pure ^{3}H-labelled OGDC.

(B) Lane 1, ^{3}H-labelled OGDC; lanes 2-5, immune precipitation of PK-15 cell extracts with pre-immune serum (lane 2); anti-OGDC serum (lane 3); E1 subunit specific antiserum (lane4) and anti-E2 serum (lane 5).


(A)
(B)
pre-E2
E1
E3
E2
1 2 3 4
1 2 3 4 5

5.4 DETECTION OF SUBUNITS OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX IN PORCINE KIDNEY CELLS LABELLED IN THE PRESENCE OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

As import of cytoplasmically-synthesised mitochondrial polypeptides is dependent on the electrochemical potential gradient across the inner membrane of the organelle, dissipation of $\Delta\mu_H+$ in the presence of uncouplers of oxidative phosphorylation can lead to the accumulation of precursor forms of the constituent enzymes of OGDC,

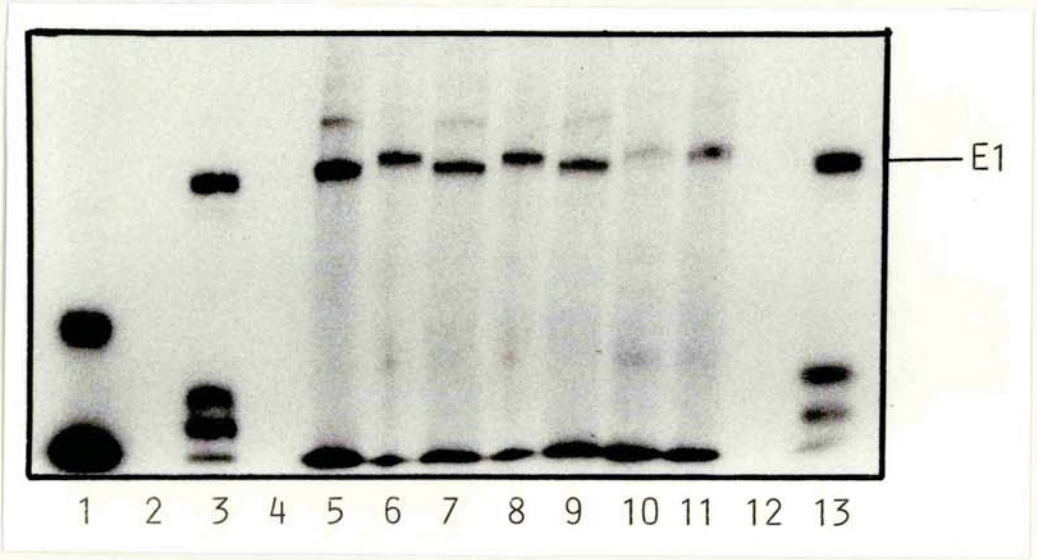
provided these are relatively stable in the cytosolic compartment of the cell.

Determination of conditions which prevented import of proteins into mitochondria was a question of trial and error. Two readily available uncouplers were 2,4-dinitrophenol (2,4-DNP) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). The latter is reported to block import of proteins into mitochondria when used in the μM range and the former in the mM range (Reid & Schatz, 1982; Fenton et al., 1984). Immune precipitation from cells, labelled with [^{35}S]methionine in the presence of 5μM FCCP revealed, in addition to the subunits of OGDC, extra bands of higher M_r value. If the concentration of FCCP was raised to 10μM the bands corresponding to the mature subunits disappeared and only the bands seen in Fig. 5.2B remained. A similar pattern was displayed in the presence of 2,4-DNP at 1mM and 2mM respectively. At the lower concentration, the mature forms were visible, in addition to the extra bands, but at the higher concentration, these had disappeared (Fig. 5.2A). Raising the 2,4-DNP concentration to 4mM resulted in decreased incorporation of

FIGURE 5.3 M_r difference of E1 isolated from porcine kidney cells labelled with [^{35}S]methionine in the presence or absence of 2,4-DNP.

Extracts prepared from cells labelled with [^{35}S]methionine in the presence or absence of 2mM 2,4-DNP were subjected to immune precipitation using E1 subunit specific antiserum. Immune products were separated on a 6% (w/v) polyacrylamide gel and visualised by fluorography.

Lane 1, M_r standards; lanes 3 & 13, ^{3}H-labelled OGDC. Immune prcipitates from cells grown minus uncoupler (lanes 5,7,9 & 11) and plus uncoupler (lanes 6,8 & 10).


E1
1 2 3 4 5 6 7 8 9 10 11 12 13

[^{35}S]methionine. Incubation of cells with [^{35}S]methionine for 2h or 4h in the presence of uncouplers gave the same result. Since greater incorporation of radioactivity into protein occurred in 4h, this period of time was used for all subsequent incubations. The total amount of [^{35}S]methionine incorporated into protein in the presence of these uncouplers was approx. 50% of that in cells labelled in the absence of uncoupler.

Several interesting conclusions can be drawn from the data in Fig. 5.2 (a) anti-E2 serum precipitates a precursor form of E2 with M_r value 6000-8000 larger than the mature subunit, (b) on 10% (w/v) SDS-polyacrylamide gels, there is no obvious difference in the M_r value of mature E1 and the product precipitated from cells grown in the presence of uncouplers by anti-E1 serum, (c) anti-OGDC serum fails to detect accumulation of pre-E2, recognising only the same component as E1-specific serum, (d) no polypeptide corresponding to E3 is observed in the final immune precipitates, again reflecting the low immunogenicity of this species and (e) precursor forms of identical M_r value are detected, regardless of the chemical nature of the uncoupler.

Fig. 5.3 demonstrates the appearance of a larger M_r precursor of the E1 subunit (pre-E1) in PK-15 cells, treated with 2,4-DNP, after separation from mature E1 on 6% (w/v) SDS-polyacrylamide gels. As 2-oxoglutarate dehydrogenase, E1, has a high intrinsic M_r value, 103500, it is difficult to observe the decreased mobility of pre-E1 on standard 10% (w/v) polyacrylamide gel systems. A combination of a lower percentage gel and increased distance of migration was employed in this experiment to achieve the necessary resolution.

A similar protocol was employed to detect the precursor forms of the component enzymes of OGDC in NBL-1 cells. The difference in M_r

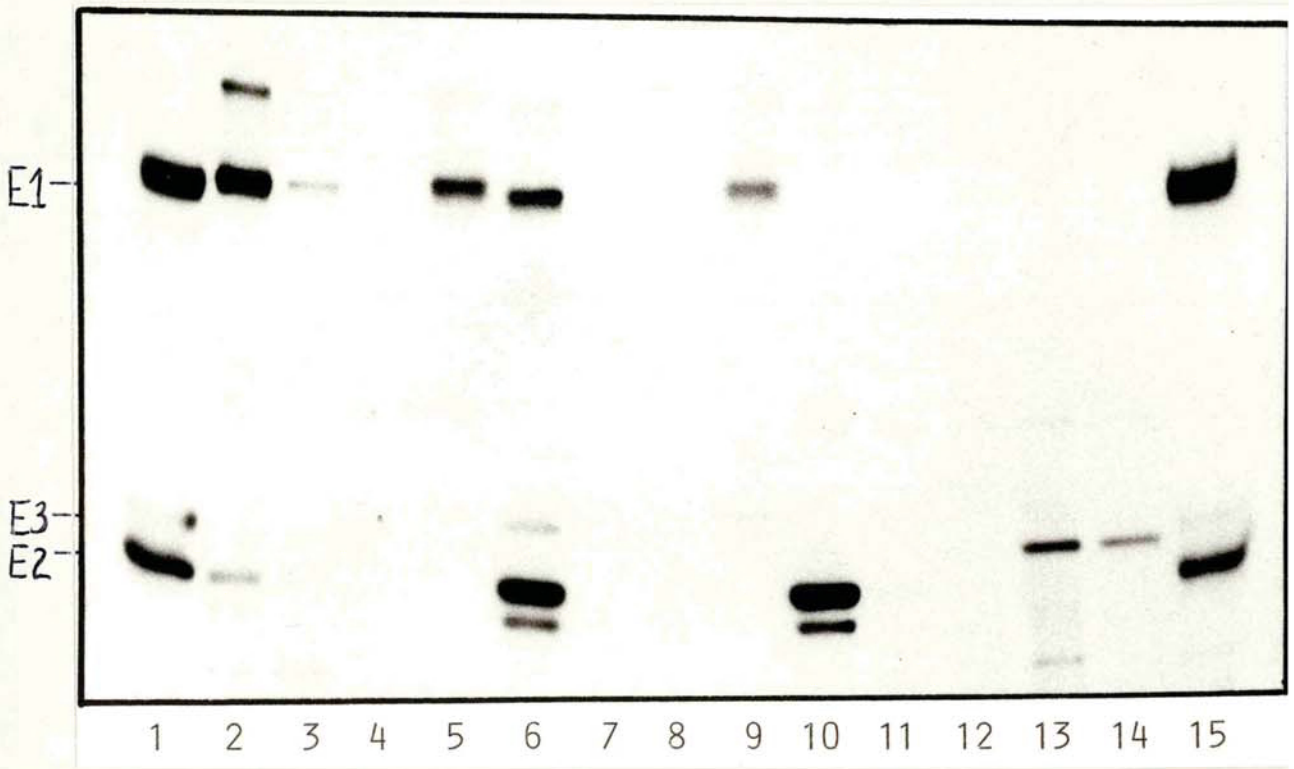
TABLE 5.1 Difference in M_r between precursor and mature forms of the component enzymes of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes in porcine kidney and bovine kidney cell lines.

COMPLEX	SUBUNIT	M_r EXTENSION SEQUENCE	
		PK-15	NBL-1
OGDC	E1	2000 ± 1000	2000 ± 1000
	E2	7000 ± 1000	6500 ± 1000
	E3	1500 ± 500	1000 ± 500
PDC(a)	E1 α	3000 ± 1000	3000 ± 1000
	E1 β	4000 ± 1000	4000 ± 1000
	E2	8000 ± 1000	8000 ± 1000
	E3	as above	

(a) Personal communication, O. L. De Marcucci

FIGURE 5.4 Immunological relationship of pre-E1 and pre-E2 to the mature subunits of mammalian 2-oxoglutarate dehydrogenase complex.

[^{35}S]methionine-labelled cell extracts were prepared from PK-15 cells grown for 4h in the presence or absence of 2mM 2,4-DNP. Immune precipitation was performed on these extracts before or after addition of competing levels of purified OGDC (10μg) or E3 (5μg). Radiolabelled products were analysed on 6% (w/v) SDS-polyacrylamide gels.
Lanes 1 & 15, ^{3}H-labelled OGDC; lanes 2-14, immune precipitates of OGDC subunits and their respective precursors with the following antisera: mature E1, anti-E1 serum (lane 2); as lane 2 plus 10μg OGDC (lane 3); pre-E1, anti-E1 serum (lane 5); as lane 5 plus 10μg OGDC (lane 4); lanes 6-9, as lanes 2-5 but with anti-OGDC serum; lanes 10-13, as lanes 2-5 with anti-E2 serum. Lane 14, cells labelled in the presence of 2mM 2,4-DNP immunoprecipitated with anti-E2 serum in the presence of 5μg E3.


E1
E3
E2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

between precursor and mature forms of the OGDC subunits in this cell line can be seen in Table 5.1. Again the difference in M_r between pre-E2 and the mature subunit is much larger than that between pre-E1 or pre-E3 and their respective mature proteins. The same pattern was observed in the case of PDC (Table 5.1).

5.5 PRECURSOR FORMS ARE IMMUNOLOGICALLY RELATED TO THE MATURE SUBUNITS OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

Confirmation of the identity of the immunoprecipitated mature polypeptides and their precursor states was achieved in competition studies, where the immunological similarity of the two forms was revealed Fig. 5.4. Immunoprecipitations were performed from [^{35}S]methionine-labelled extracts of PK-15 cells with anti-OGDC, anti-E1 and anti-E2 sera to yield the expected pattern of immune precipitation (Fig. 5.1). If non-radioactive purified OGDC (10μg) was added to the extracts, prior to analysis, the radiolabelled bands corresponding to mature E1 and E2 were excluded from the final immune precipitate. A similar immunological relationship was illustrated, when [^{35}S]methionine-labelled pre-E1 and pre-E2 also disappeared from the final immune complex, if immune precipitation was conducted in the presence of pure OGDC. This suggests strongly that the precursor forms of E1 and E2 are immunologically related to the native polypeptides.

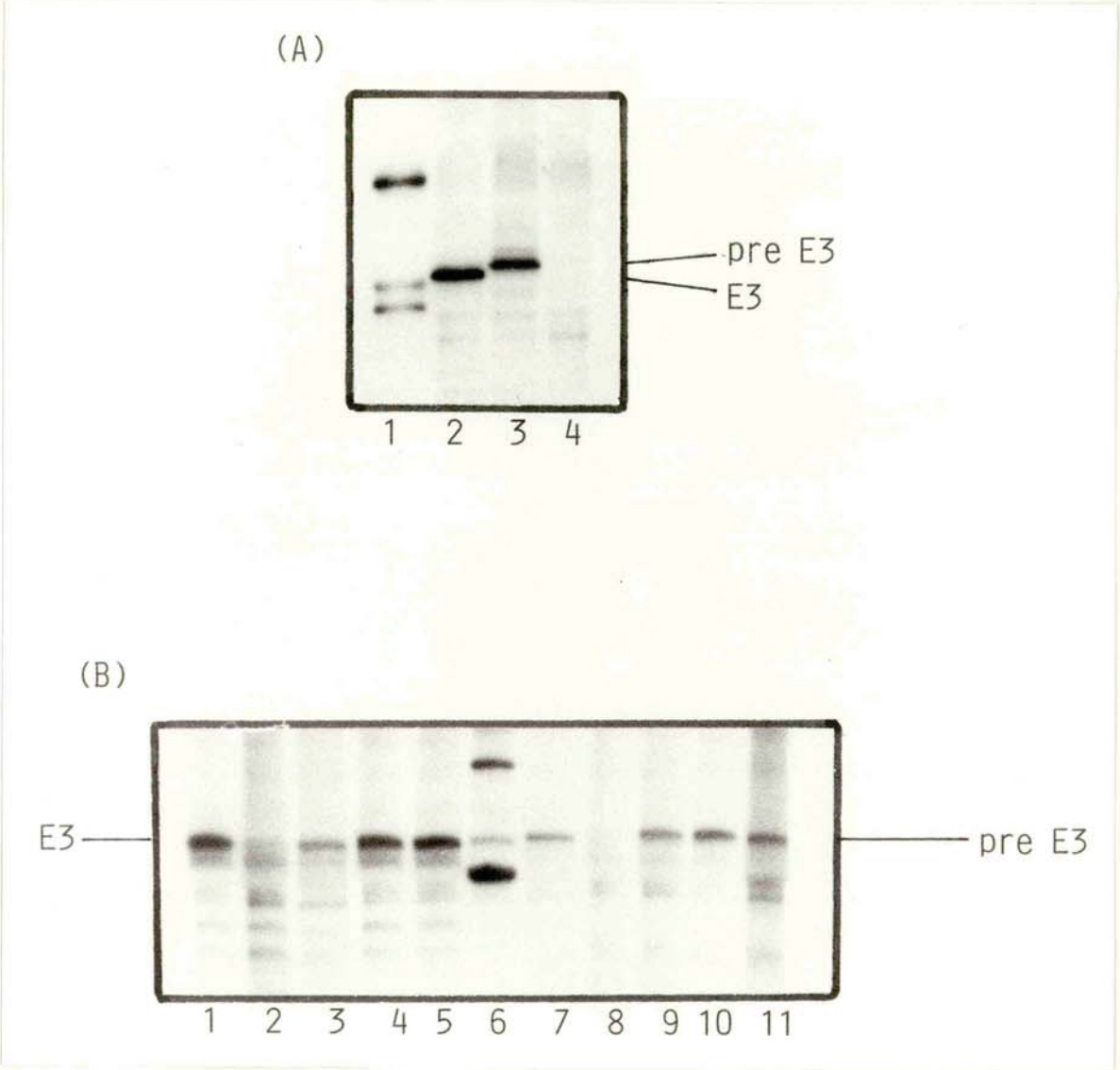
In the case of pre-E2 which has an apparent M_r value of 54500-56000, almost co-migrating with E3, M_r 54500 on SDS-polyacrylamide gels, it was also possible to confirm that this species was not the E3 subunit which may have been immune-precipitated by virtue of its association with the E2 'core' enzyme. On specific immune

FIGURE 5.5 Purified lipoamide dehydrogenase competes with pre-E3 in immune precipitation studies.

Anti-E3 serum was employed in immune precipitation of [^{35}S]methionine-labelled extracts of PK-15 cells, grown in the presence or absence of 2mM 2,4-DNP (A). In addition, immune precipitation was performed from these extracts with anti-E3 serum in the presence of competing amounts of purified porcine heart E3, OGDC or PDC purified from bovine heart or pure BCDC from bovine kidney (B).

(A) Lane 1, ^{3}H-labelled OGDC; lane 2, E3 from cells grown minus uncoupler; lane 3, E3 from cells grown in the presence of uncoupler; lane 4, as lane 3 with preimmune serum.

(B) Lanes 1-5, immune precipitation of E3 from cells grown minus uncoupler in the presence of no competition (lane 1); 5µg purified E3 (lane 2); 10µg pure OGDC (lane 3); 10µg PDC (lane 4) and 10µg pure BCDC (lane 5); lane 6, cells grown minus uncoupler immune precipitated with anti-OGDC serum. Lanes 7-11, as lanes 1-5 but from cells grown in the presence of 2,4-DNP.


(A)
pre E3
E3
1 2 3 4
(B)
E3
pre E3
1 2 3 4 5 6 7 8 9 10 11

precipitation of pre-E2 with anti-E2, no competing effects were observed after addition of pure lipoamide dehydrogenase (E3) to the cell extracts, confirming that E3 and the higher M_r precursor of E2 were separate molecules of similar size which are immunologically distinct.

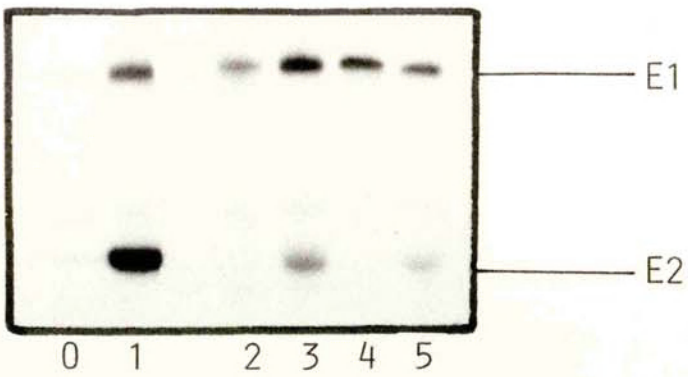
Fig. 5.5A reveals that anti-E3 serum precipitates a precursor form of E3 with M_r value 1000-2000 larger than the mature subunit from [^{35}S]methionine-labelled detergent extracts of PK-15 cells, grown in the presence of 2,4-DNP. The immunological relationship of this precursor to the mature E3 subunit of OGDC was confirmed by immunocompetition studies where, addition of purified porcine heart E3 (5μg) to the extracts, prior to immune precipitation, resulted in both mature (lane 2) and precursor (lane 8) forms of E3 being excluded from the final immune complex (Fig. 5.5B).

An interesting finding in these immunocompetition studies arose when competing levels of purified bovine heart OGDC and PDC (10μg) or bovine kidney BCDC (10μg) were added to the extracts before the addition of the anti-E3 serum. Previous immune blotting analysis had revealed that the antiserum did cross react with the E3 components of OGDC and PDC, while E3 was absent from purified BCDC (Fig. 4.9). Therefore it was expected that OGDC and PDC but not BCDC would compete with E3 in crude extracts for the antiserum and that E3 would therefore be absent from the final immune precipitates. As can be seen from Fig. 5.5B, none of the three 2-oxo acid dehydrogenase complexes were effective in competing out the E3 polypeptide.

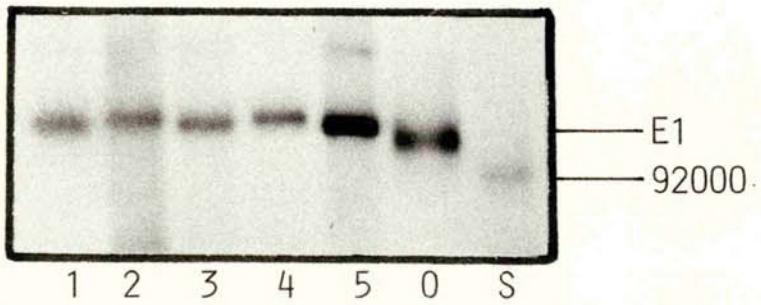
This could be explained by assuming that, although bovine heart E3 has determinants in common with porcine heart E3 (the original

FIGURE 5.6 Precursor forms of the component enzymes of 2-oxoglutarate dehydrogenase complex can be chased to the mature forms on removal of uncoupler.

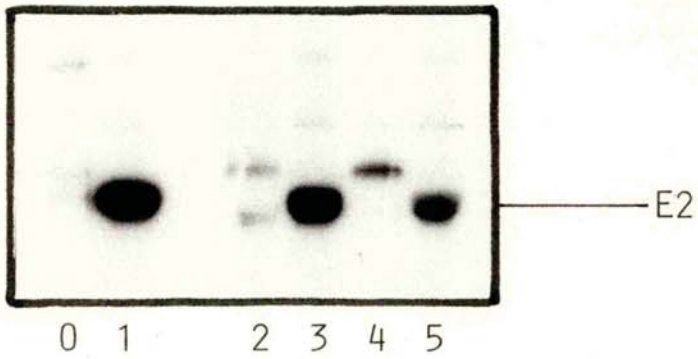
Cells were labelled with [^{35}S]methionine for 4h either in the presence or absence of uncoupler. After this time the cells were either lysed as described in section 2.2.8 a, or the radioactive medium was replaced with normal growth medium and the cells incubated for another 40 min at 37^{o}C before extracts were prepared. Extracts were then incubated with antisera against native OGDC (A), E1 (B), E2 (C) or E3 (D). Immune precipitated material was separated on (A & C) 10% (w/v) or (B & D) 6% (w/v) SDS-polyacrylamide slab gels and detected by fluorography. Lane O, ^{3}H-labelled OGDC. Lane S, M_r standards. Lane 1, 4h label, no uncoupler; lane 2, 4h label in presence of 2mM 2,4-DNP; lane 3, as lane 2 with 40 min chase in NGM; lane 4, 4h label in presence of 10μM FCCP; lane 5, as lane 4 with 40 min chase.



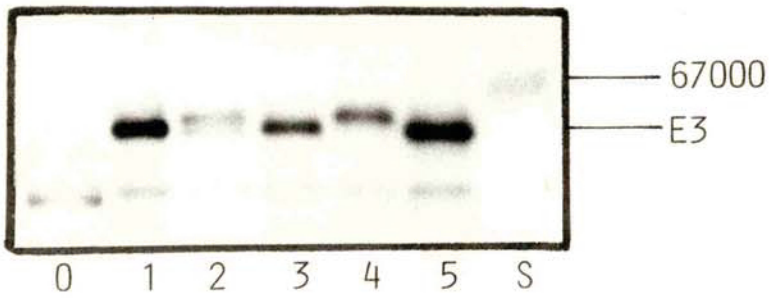
(B)



(C)



(D)



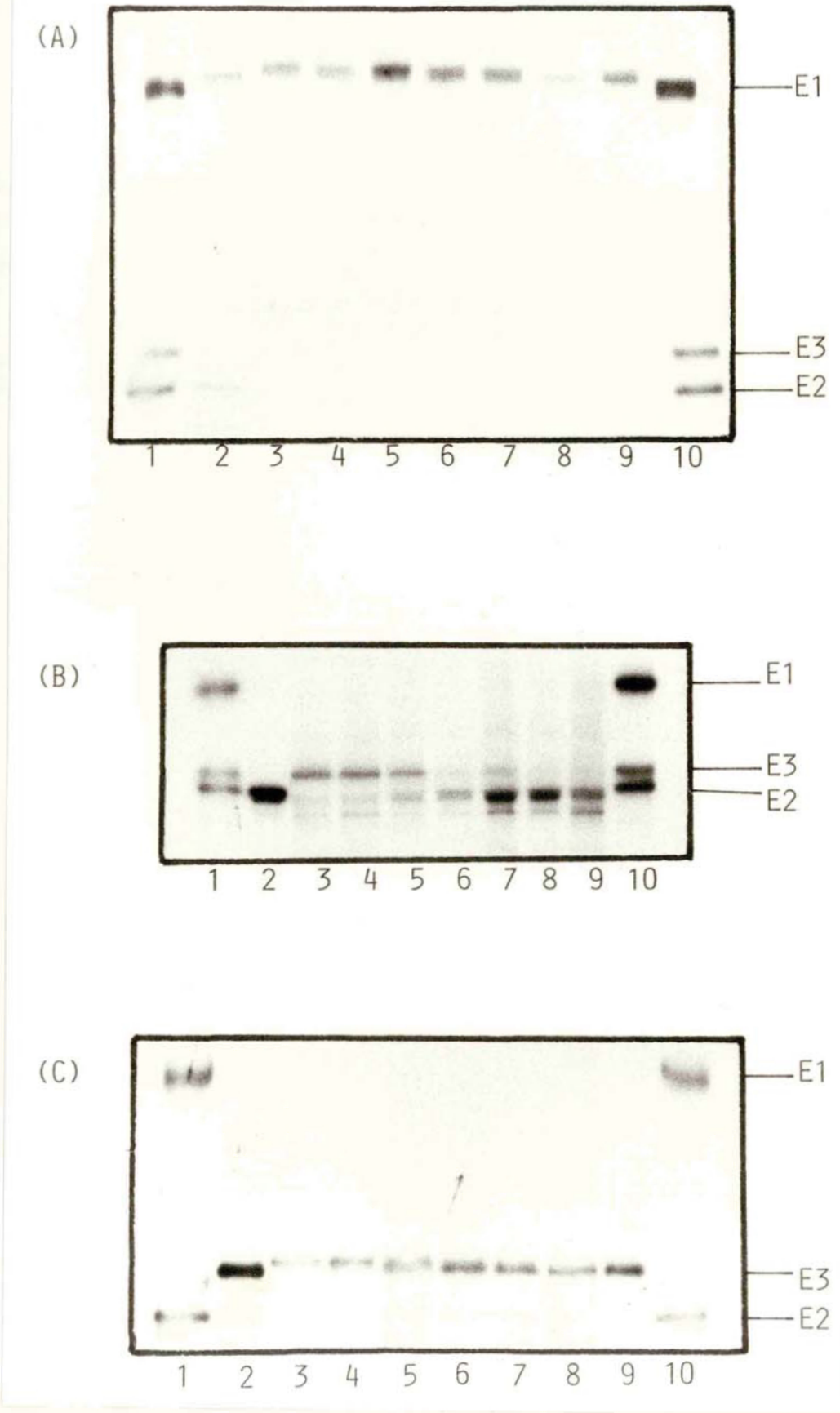
antigen), there are some determinants shared between porcine heart and porcine kidney E3 which are absent from the equivalent bovine heart enzyme. At least two pieces of evidence have been presented to substantiate this theory. There must be common determinants between the original antigen and bovine heart E3 in order to explain the cross reaction seen in immunoblotting analysis (Fig. 4.9). In addition, there must be common determinants between porcine kidney E3 and the antigen to account for the ability of the antisera to immunoprecipitate E3 from PK-15 cells. If both porcine kidney and bovine heart E3 are present, with the latter in excess, then bovine heart E3 will compete for antibody raised against the determinants common to itself and the antigen. However, if there are additional determinants on the antigen, not present on bovine heart E3 but present on porcine kidney E3, then antibodies to these determinants would not be competed out by bovine heart E3 and would still be free to react with E3 of OGDC in PK-15 cells. In this case only if OGDC, PDC and BCDC, purified from porcine heart, were used in the competition experiments, assuming that the same E3 is common to all three complexes, would efficient competition result.

5.6 CONVERSION OF PRECURSORS TO THE MATURE FORMS OF THE SUBUNITS OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

Precursor forms of the component enzymes of OGDC, accumulated for 4h in the presence of 2,4-DNP or FCCP, are rapidly processed to the mature form on removal of either uncoupler, indicating a reversible mode of action of these agents (Fig. 5.6). The conversion to mitochondrially located mature subunits in this 'pulse-chase'

FIGURE 5.7 Time-course of processing of precursors to the component enzymes of the 2-oxoglutarate dehydrogenase complex.

PK-15 cells were labelled with [^{35}S]methionine for 4h in the presence of 2mM 2,4-DNP. Radioactive medium was replaced with normal growth medium and the cells incubated for further 2-40 min before being lysed with detergent. Extracts were immune precipitated with E1 (A), E2 (B) or E3 (C) subunit specific antisera and products were analysed on (B) 10% (w/v) or (A & C) 6% (w/v) SDS-polyacrylamide slab gels.
Lanes 1 & 10, ^{3}H-labelled OGDC; lane 2, 4h label no 2,4-DNP; lanes 3-9, 4h label chase for 0, 2 5, 10, 15, 25 and 40 min respectively.

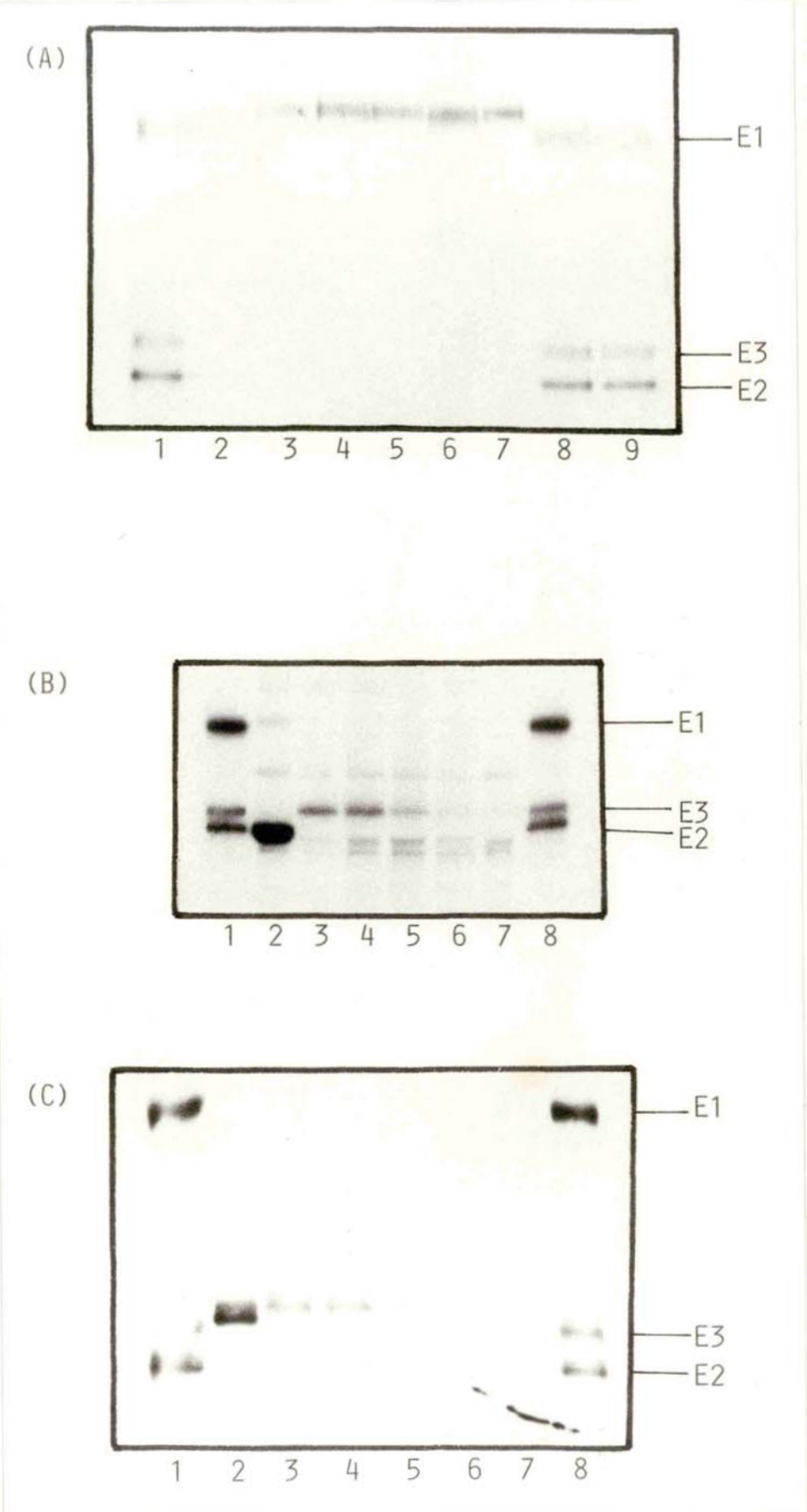

(A)
E1
E3
E2
1 2 3 4 5 6 7 8 9 10
(B)
E1
E3
E2
1 2 3 4 5 6 7 8 9 10
(C)
E1
E3
E2
1 2 3 4 5 6 7 8 9 10

procedure is monitored efficiently with the subunit specific antisera which are capable of immune precipitation of both precursors and mature enzymes. In contrast, as already seen in Fig. 5.2, the polyclonal serum to the native multienzyme complex fails to recognise E2 precursor molecules. However, after a 40min chase in the presence of non-radioactive methionine, it can detect the appearance of a component of M_r 48500, corresponding to the processed (mature) E2 polypeptide.

Subunit specific antisera were employed to follow the time course of processing on removal of 2,4-DNP (Fig. 5.7). Owing to the slight difference in M_r between the precursor and mature forms of both E1 and E3, it was difficult to find conditions which would allow separation of both forms when run in the same track on SDS-PAGE. Longer gels of lower percentage, 6% (w/v), acrylamide were run and separation of pre-E3 and the mature protein was achieved (Fig. 5.7C, lane 5). From this it can be seen that processing had begun 5 min after removal of uncoupler and although it is difficult to determine the exact time at which it was completed, all precursor had been converted to mature by 40 min after replacement of radioactive medium with NGM. Even under these conditions of longer 6% (w/v) polyacrylamide gels, pre-E1 and the mature subunit could not be easily resolved. Gels containing 6% (w/v) acrylamide with various degrees of crosslinking were tried with no further success (not shown). The band on the autoradiograph corresponding to E1 seems to migrate faster or slower depending on the expected relative amounts of pre-E1 and E1 present. In the initial stages, its mobility is similar to that of the precursor, while later comparison of the mobility of the band in adjacent tracks reveals that it describes a curve, culminating in the 40 min time point which runs

FIGURE 5.8 Stability of precursors to component enzymes of 2-oxoglutarate dehydrogenase in porcine kidney cells maintained in the presence of 2mM 2,4-DNP.

PK-15 cells were labelled with [^{35}S]methionine for 4h in the presence of 2mM 2,4-DNP. Radioactive medium was replaced by NGM supplemented with 2mM 2,4-DNP and the cells incubated for a further 0.5-4.5h. Cell extracts were incubated with anti-E1 (A), anti-E2 (B) or anti-E3 (C) sera. Immune products were resolved on (B) 10% (w/v) or (A & C) 6% (w/v) SDS-polyacrylamide gels which were then processed for fluorography.
Lanes 1 & 8, ^{3}H-labelled OGDC; lane 2, 4h label no 2,4-DNP; lanes 3-7, 4h label chase for 30 min, 1h, 2h, 3h and 4.5h respectively in the presence of 2,4-DNP.


(A)
E1
E3
E2
1 2 3 4 5 6 7 8 9
(B)
E1
E3
E2
1 2 3 4 5 6 7 8
(C)
E1
E3
E2
1 2 3 4 5 6 7 8

with the same mobility as mature E1. All that can be concluded from these studies is that processing is complete within 40 min after removal of uncoupler.

In comparison the conversion of pre-E2 to its mature form is followed with relative ease on 10% (w/v) SDS-polyacrylamide gels. As time after removal of uncoupler progresses, the amount of precursor decreases with a parallel increase in the amount of mature protein (Fig. 5.7B). Determination of the initiation of processing is complicated by the co-precipitation of two protein bands, the higher of which overlaps that of the mature subunit. However, it is certainly well underway by 10 min and completed by 40 min after removal of 2,4-DNP.

5.7 STABILITY OF PRECURSOR FORMS OF THE COMPONENT ENZYMES OF 2-OXOGLUTARATE DEHYDROGENASE COMPLEX IN PORCINE KIDNEY CELLS MAINTAINED IN THE PRESENCE OF 2,4-DNP

In order to determine the stability of the precursor forms of the component enzymes of OGDC an alternative protocol to the 'pulse-chase' procedure was followed. [^{35}S]methionine-labelled precursors were accumulated for 4h in the presence of 2,4-DNP. Radioactive medium was then replaced by NGM supplemented with 2mM 2,4-DNP and the cells incubated for various times at 37^{o}C (section 2.2.5 e).

In this case, since uncoupler is present throughout, no processing of precursor to mature forms can take place. Pre-E1 is seen to be the most stable of the three precursors (Fig. 5.8A), being present 4.5h after the removal of radioactive methionine. In the cases of pre-E2 (Fig. 5.8B) and pre-E3 (Fig. 5.8C), the precursor forms

start to disappear 2h after addition of the non-radioactive medium and very little, if any, is still present after 4.5h.

5.8 LOCATION OF PRE-E2 IN THE CELL

As stated earlier (section 1.3.2), the determination of the intracellular site of synthesis of mitochondrial precursors is central to the question of how mitochondria import proteins. The majority of mitochondrial precursors studied to date are located in the cytosol of the cell.

Cells were treated with digitonin in order to prepare soluble and particulate fractions. Digitonin disrupts cellular membranes by interaction with cholesterol in these membranes. The high cholesterol content of the plasma membrane in comparison to the mitochondrial inner membrane allows conditions to be determined whereby the cells are lysed but mitochondria remain intact.

In order to determine the correct levels to use, cells were treated with various levels of digitonin (see section 2.2.8 b) and activities of fumarase and citrate synthase in the soluble fractions were assayed (see section 2.2.1). Citrate synthase is a mitochondrial matrix enzyme and the presence of its activity in the soluble fraction was an indication of mitochondrial disruption. Fumarase has a dual location in the cell, being present both in the cytosol and the mitochondrial matrix. Activity of fumarase would be expected to rise initially as cells are broken, then plateau as all cells are lysed but no mitochondria are broken i.e. cytosolic fumarase is released. A further increase in fumarase activity which should parallel the rise

FIGURE 5.9 Activity of mitochondrial matrix enzymes in the cytosol of porcine kidney cells treated with digitonin.

Soluble fractions from cells treated with 0, 0.25, 0.5, 1.0, 2.0, 5.0 or 10.0mg/ml digitonin as described in section 2.2.8 were assayed for fumarase (section 2.2.1 c) citrate synthase (section 2.2.1 b) and protein (section 2.2.2) Enzyme activity was then plotted against mg digitonin/mg protein.

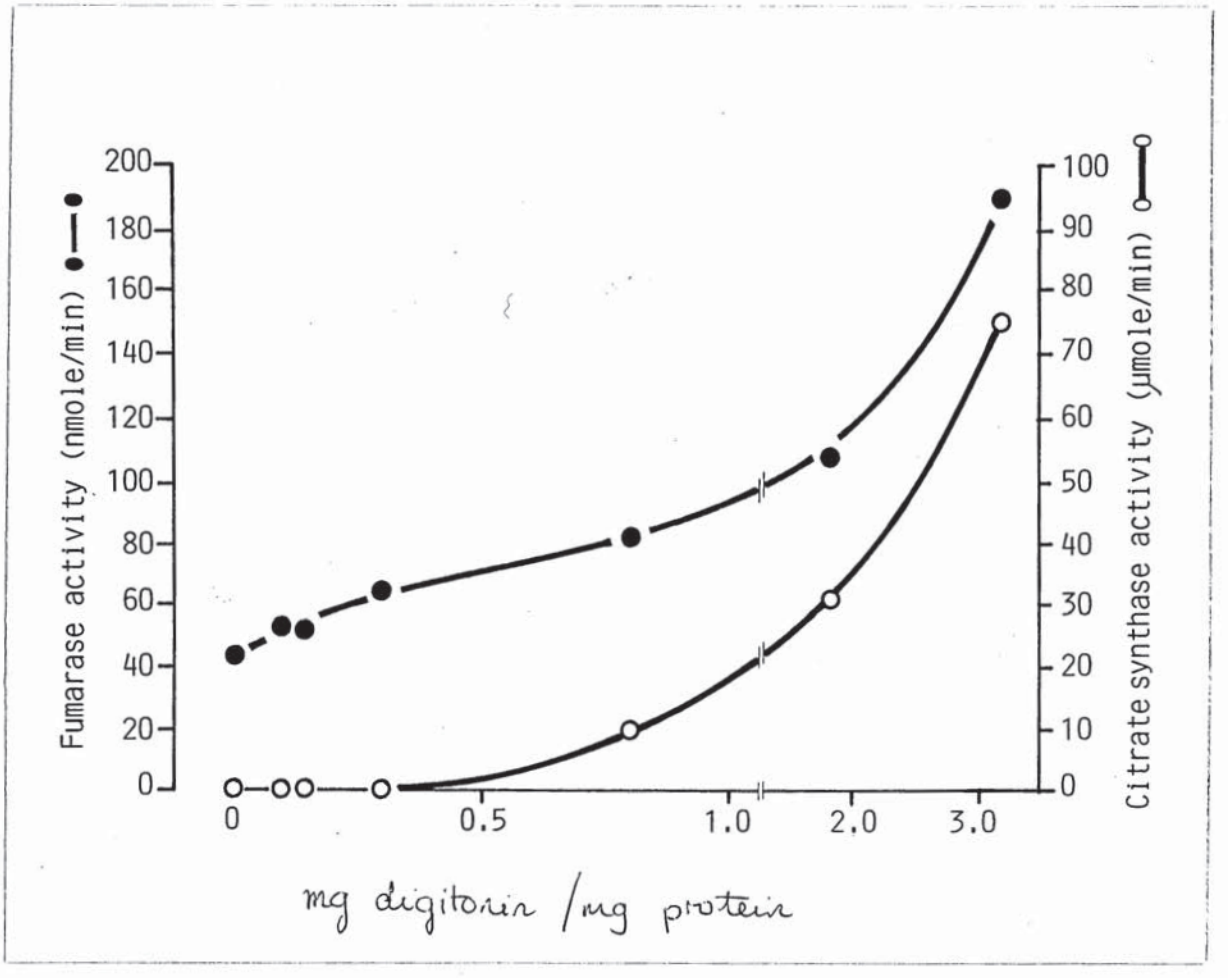

Fumarase activity (nmole/min)
200
180
160
140
120
100
80
60
40
20
0
Citrate synthase activity (μmole/min)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1.0
2.0
3.0
mg digitonin / mg protein

FIGURE 5.10 Reactivity of citrate synthase antiserum with cytosolic fractions from porcine kidney cells treated with various levels of digitonin.

Soluble fractions were obtained from PK-15 cells treated with various levels of digitonin as described in section 2.2.8 Samples of these fractions were resolved on 10% (w/v) SDS-polyacrylamide slab gels. One gel was stained with Coomassie blue to detect protein bands (A) and the polypeptides on the other gel were transferred to nitrocellulose and then probed with citrate synthase antiserum (1:100 dil.) to detect antigenic proteins (B). Lane 1, M_r standards; lanes 2 & 11, 5µg pure citrate synthase; lane 3, PK-15 mitochondria (40µg in A; 75µg in B); lanes 4-10, cytosolic fractions from cells treated with 10.0, 5.0, 2.0, 1.0, 0.5, 0.25 and 0mg/ml digitonin respectively (40µg in A; 75µg in B).

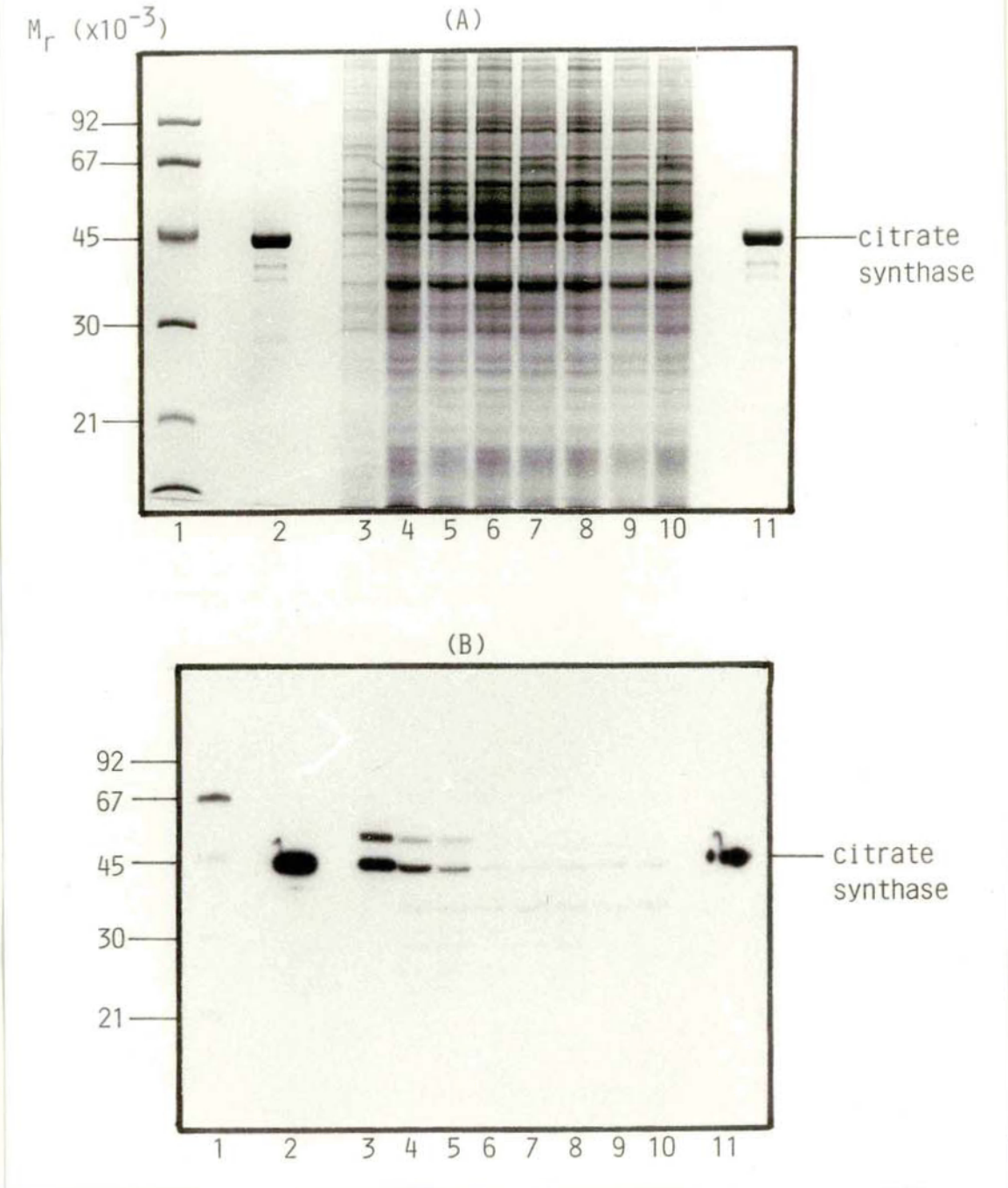
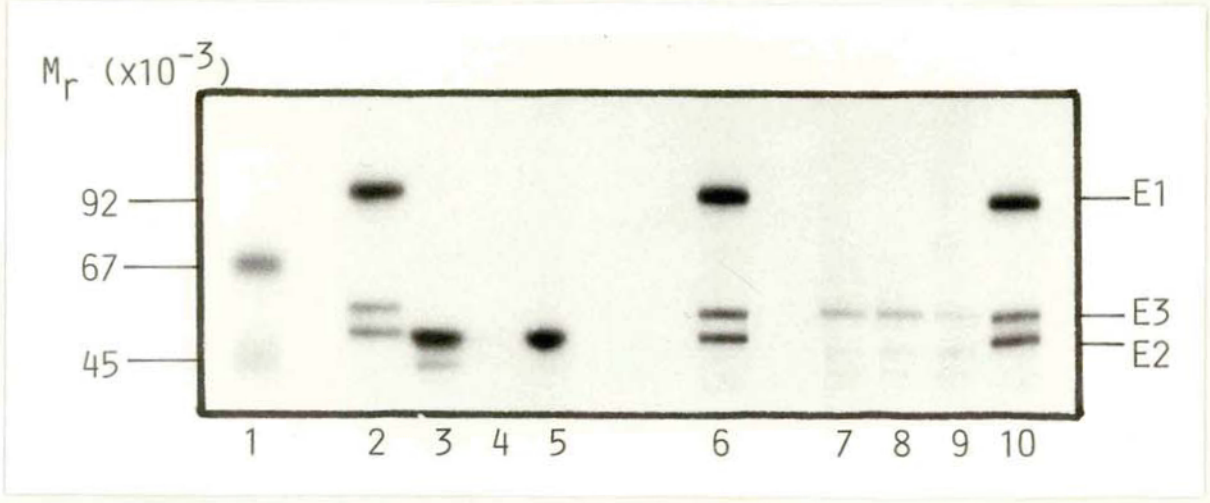

(A)
M_r (x10^{-3})
92
67
45
30
21
1 2 3 4 5 6 7 8 9 10 11
citrate synthase
(B)
92
67
45
30
21
1 2 3 4 5 6 7 8 9 10 11
citrate synthase

FIGURE 5.11 Pre-E2 is located in the cytosol of porcine kidney cells.

PK-15 cells labelled for 4h with [^{35}S]methionine in the presence or absence of 2mM 2,4-DNP were extracted with detergent as normal, or treated with 0.5mg/ml digitonin to prepare soluble and particulate fractions. Immune precipitation was performed with anti-E2 serum and the products analysed on a 10% (w/v) SDS-polyacrylamide gel.
Lane 1, M_r standards; lanes 2, 6 & 10, ^{3}H-labelled OGDC. Lanes 3-5, PK-15 cells grown in the absence of uncoupler; lane 3, cell extract; lane 4, soluble fraction; lane 5, particulate fraction. Lanes 7-9, PK-15 cells labelled in the presence of 2,4-DNP and extracted with detergent (lane 7) or separated into soluble (lane 8) and mitochondrial (lane 9) fractions.


M_r (x10^{-3})
92
67
45
E1
E3
E2
1
2
3
4
5
6
7
8
9
10

in citrate synthase activity would indicate mitochondria were being disrupted.

Fig. 5.9 reveals that even at 0mg digitonin/mg protein, fumarase activity can still be detected. This is probably due to mechanical breakage of the cells during the harvesting from petri-dishes and subsequent vortexing. Citrate synthase activity was not detected until digitonin levels of 0.4mg/mg protein were employed.

Soluble fractions from these digitonin-treated cells were resolved by SDS-PAGE and probed with anti-citrate synthase antiserum (a gift from O. L. De Marcucci). The immunoblotting technique is probably a more sensitive indication of the appearance of citrate synthase i.e. disruption of mitochondria and was used to confirm the result of Fig. 5.9 involving enzymic activity measurements. A low level of citrate synthase activity was observed in all fractions while a significant increase in the amount of citrate synthase present is seen when 0.8-1.0mg digitonin/mg protein is employed (Fig. 5.10, lanes 6 & 5 respectively). It was concluded that digitonin levels of < 0.5mg/mg protein could break open cells and keep mitochondrial disruption to a minimum. Digitonin levels used to treat [^{35}S]methionine-labelled cells were 0.5mg/ml or 0.25mg/dish i.e. in the region 0.1-0.3mg digitonin/mg protein.

When immune precipitations were performed from whole cells, soluble and particulate fractions prepared from [^{35}S]methionine-labelled cells by digitonin treatment, mature E2 was found to be located almost exclusively in the particulate fraction (Fig. 5.11). The small percentage of E2 in the soluble fraction can be attributed to breakage or leakiness of some mitochondria. In contrast, immune precipitations from soluble and particulate fractions, prepared from cells labelled

with [^{35}S]methionine in the presence of 2,4-DNP, revealed that pre-E2 was located almost solely in the cytosolic fraction. A small amount of precursor was seen in the particulate fraction and may represent binding of pre-E2 to receptors on the surface of mitochondria.

On trypsin treatment of these extracts prior to immune precipitation, precursor present in both soluble and particulate fractions was digested while mature E2 in the particulate fraction was protected from degradation (not shown). In addition, after a chase period of 40 min, pre-E2 had been converted to the mature protein which was found in the particulate fraction (not shown).

The conclusion drawn from this result is that pre-E2 is synthesised in the cytoplasm. It is likely that the other precursors are also located in this compartment although this was not tested.

5.9 DISCUSSION

Considerable effort was expended in determining optimal conditions for labelling cells with [^{35}S]methionine and in obtaining reproducible immunoprecipitates of the component enzymes of OGDC with minimal background from contaminating polypeptides. Most of the protocols reported in the literature rely on detergent mixtures containing various combinations of SDS and Triton X-100 in which cells are often treated initially with hot SDS to eliminate possible proteolysis of precursor polypeptides (Ohashi *et al.*, 1982; Gasser, 1983). Unfortunately, these protocols involve nuclear lysis, releasing DNA into the supernatant fractions, with the attendant problems of contamination of immune precipitates described earlier (section 5.2).

Our strategy involves initial solubilisation of cells and rapid

removal of nuclei. Proteolysis is kept to a minimum by carrying out all procedures at 4°C and by including 1mM 1,10-phenanthroline, an inhibitor of the matrix protease responsible for maturation of precursors, and 1mM benzamidine-HCl, a general protease inhibitor, in the buffers. In addition all buffers were sterilised by passage through Nalgene filters, pore size 0.20μm, prior to use. This reduced the risk of contamination of the extracts with bacterial protein or degradation by bacterial proteases.

The buffer system employed for these immune precipitations contained 1% (v/v) Triton X-100, 0.5% (w/v) SDS and 1% (w/v) DOC, and although the yield of precipitated antigen was lower than in other buffers (Fig. 4.11), the stringent washing conditions resulted in excellent backgrounds in control samples.

Immune precipitation of mature E1 and E3 revealed that in porcine kidney cells the apparent M_r value of these subunits was higher than the corresponding bovine heart enzymes (Figs. 5.5; 5.6; 5.7 & 5.8).

Data presented here clearly demonstrated that all three enzyme components of OGDC were synthesised as larger precursor molecules in these cell lines. This contrasts with the results of Matuda & Saheki (1982) who reported that the primary translation product of rat liver lipoamide dehydrogenase had the same M_r value as the mature E3. However, the authors conceded that a larger precursor could have been present but was not detected due to rapid degradation by endogenous proteases or because the size difference was so small that it could not be detected on 10% (w/v) polyacrylamide gels. In agreement with the latter point, it was difficult to differentiate between pre-E3 and mature E3 on 10% (w/v) gels using fluorographic analysis which is

probably more sensitive to small differences in M_r than the gel slicing technique employed by Matuda & Saheki (1982). Indeed, in a later paper, the same authors (Matuda et al., 1983) claim that E3 of rat liver is initially synthesised as a precursor form which is approximately 3000 larger in M_r than the mature E3. This second study is concerned with immune precipitation of E3 from the translation products of a cell-free system primed with rat liver mRNA. However direct comparison of mature and precursor forms of E3 on SDS-polyacrylamide gels was not carried out.

It is difficult to make an accurate determination of the absolute M_r of pre-E1 and pre-E2. In the case of E1 this is because of the difficulty in determining M_r in the region 100000-200000 by SDS-PAGE. Pre-E2, however, exhibits a differential mobility on 10% (w/v) and 6% (w/v) polyacrylamide gels. As seen in Fig. 5.2, pre-E2 appears to have a marginally lower mobility than mature bovine heart E3 whereas on 6% (w/v) polyacrylamide gels, this polypeptide appears to migrate faster than E3.

The available evidence also suggests that the E2 precursor, at least, is located in the cytosol of the cell on completion of translation. Matuda et al. (1983) reported that E3 was synthesised on free ribosomes suggesting that pre-E3 would also be located in the cytosol.

It is fortunate that the precursor polypeptides are relatively stable, remaining in the cytosol for several hours if the cells are maintained in the presence of uncoupler. In some cases, it has been reported that precursor forms are degraded rapidly if normal import is prevented in this manner (Raymond & Shore, 1981).

Complete reversal of uncoupler action was achieved by incubation

of the cells in fresh medium. Each individual activity, synthesised separately as an individual precursor protein, was converted to the mature form on removal of the uncoupler. The lag phase observed before initiation of maturation could be the time required for re-energisation of mitochondria. A similar lag between reversal of uncoupler action and import of protein was observed in rho$^-$ yeast cells labelled with [^{35}S]methionine in the presence of the uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Reid & Schatz, 1982).

Two points of interest emerged in relation to the structure of these mitochondrial precursor molecules. It was apparent that pre-E2 was not recognised to any significant extent by antibody to native OGDC. On occasions, a faint band was observed, in immune precipitations of cells labelled in the presence of 2,4-DNP, with M_r approximately equal to E3 but it was impossible to tell whether this was small amounts of pre-E2 or small amounts of pre-E3 due to the relatively low titre of antibody to E3. The presence of pre-E2 was monitored easily with subunit specific antiserum, prepared against the denatured polypeptide. As it is a major assumption in these experiments that antisera raised to the mature protein will cross react with the precursor, it is fortunate that antiserum to denatured E2 was also produced.

Similar effects have been noted previously e.g. antibody to apocytochrome c fails to recognise the holocytochrome and vice versa (Hennig & Neupert, 1981). In addition, antibody to ATPase subunit IX exhibits only a very weak cross reaction with the precursor form of this protein (Schmidt *et al.*, 1983b)

A plausible interpretation of these data is that the conformation

of pre-E2 is so different from that of the mature form that it is recognised only weakly or not at all by antibody raised to the native E2 component of OGDC.

Recent studies on the nature of antigenic determinants in proteins have suggested that considerable proportions of these antigenic determinants are discontinuous in nature (Todd et al., 1982; Westhof et al., 1984). Thus, they consist of specific regions of the protein which exist in a defined three-dimensional conformation often composed of non-continuous segments of amino acid sequence in close proximity. In contrast, for the denatured protein, it is short regions of primary sequence (5-8 amino acids) which are thought to provoke the dominant antibody response. Such an interpretation would account for our present observations, confirming that pre-E2 and mature E2 have many sequences in common, while existing in dissimilar conformational states.

Another point of interest relates to the size of the signal sequence of pre-E2, which has an M_r value of 6000-8000, in comparison to those of pre-E1 and pre-E3 which are only 1000-3000. In relation to this point, the signal sequence of the precursor to the lipoate acetyltransferase (E2) core of mammalian PDC is much larger than that for the other subunits (Table 5.1).

The signal sequence on E2 may not be required solely for targetting this polypeptide to mitochondria but, in addition, may also contain elements necessary to maintain the precursor in a conformation distinct from the mature protein, thereby preventing premature aggregation into the core assembly before import into the organelle.

CHAPTER SIX

DISCUSSION

6.1 DISCUSSION

It is now clear that the limited coding potential of the mitochondrial genome necessitates that the vast majority of mitochondrial polypeptides are nuclear-coded, and must be synthesised on cytoplasmic ribosomes, usually as larger M_r precursor forms containing an NH_2-terminal extension (Hay et al., 1984). Subsequent uptake into the organelle is a posttranslational event, which is dependent on the presence of an electrochemical gradient ($\Delta\mu_H+$) across the mitochondrial inner membrane (Gasser et al., 1982a; Schleyer et al., 1982). Proteolytic cleavage of these higher M_r precursors is carried out by a neutral, chelator-sensitive, matrix protease (Mori et al., 1980; McAda & Douglas, 1982; Bohni et al., 1983; Schmidt & Neupert, 1984). The final stage in the import process, following translocation and processing, is assembly of newly-synthesised polypeptides into functional units inside the mitochondrion. In comparison to other steps in the pathway, there have been relatively few reports on assembly of mitochondrial proteins into functional complexes. Another area of mitochondrial biogenesis where information is lacking is that of attachment of cofactors or prosthetic groups to mitochondrial proteins.

Unlike the problems of determination of sequences responsible for targetting proteins to mitochondria and identification of receptor molecules responsible for specific binding of mitochondrial precursors, which are probably best tackled in systems such as yeast using genetic manipulation and recombinant DNA technology, the question of cofactor attachment and assembly of multiprotein complexes can be addressed in mammalian cells.

A good candidate for studies relating to these problems is the 2-oxoglutarate dehydrogenase complex which is a mitochondrial matrix assembly composed of three different enzyme subunits. Five coenzymes and prosthetic groups are involved in the sequence of reactions catalysed by this multiprotein aggregate: thiamine pyrophosphate, lipoic acid, CoA, FAD and NAD^+. A survey of the literature has revealed that, although the structural, functional and regulatory properties of this key multienzyme complex have been thoroughly investigated over the years (Koike & Koike, 1976; Roche & Lawlis, 1982), no detailed studies on the biogenesis, translocation and assembly have been performed to date. Examination of the biosynthesis of OGDC also has the advantage of the possibility of comparison with the other 2-oxo acid dehydrogenase complexes (PDC & BCDC) which are structurally and functionally analogous to OGDC.

Bovine heart was chosen as the source for purification of OGDC as a rapid procedure giving a good yield of pure enzyme had been well described by Stanley & Perham (1980). High titre, monospecific, polyclonal antisera were raised to intact, native OGDC and its component enzymes, 2-oxoglutarate dehydrogenase (E1), lipoyl succinyltransferase (E2) and lipoamide dehydrogenase (E3). Specific immune precipitation, employing these antisera, revealed that all three subunits of OGDC were initially synthesised as larger M_r precursors in PK-15 and NBL-1 cells. These higher M_r forms were identified in cells labelled with [^{35}S]methionine in the presence of uncouplers of oxidative phosphorylation. This protocol was employed in preference to attempting to detect the precursor molecules in cells labelled for a very short time with [^{35}S]methionine. In the latter situation, there are problems of incorporating sufficient radioactivity into protein

and also of short half-lives of precursor molecules in the extra-mitochondrial pool. In the presence of uncouplers of oxidative phosphorylation, the electrochemical gradient across the mitochondrial inner membrane is dissipated, import of proteins into the organelle is inhibited and therefore precursors accumulate in the cytoplasm. It is fortunate that the precursor forms of the component enzymes of OGDC are stable for several hours in cells maintained in the presence of 2,4-DNP, allowing their detection with highly specific antisera.

The presence of these precursor forms in the cytosol, several hours after the removal of radioactive methionine, is in agreement with results obtained by Fenton et al. (1984) who observed pre-methylmalonyl-CoA mutase in BRL cells after a 5h chase in the presence of 2,4-DNP. This is, however, in complete contrast to the results obtained for pre-carbamylphosphate synthase (Raymond & Shore, 1981) and pre-aspartate aminotransferase (Jaussi et al., 1982). In both cases, when processing or translocation was blocked, the respective precursors disappeared within 10 min. This unequal stability of precursor molecules was also observed in rho^- mutants of S. cerevisiae (Reid & Schatz, 1982) maintained in the presence of uncoupler. Precursor forms of both the α and β subunits of F_1-ATPase were found to be considerably more stable than the precursor of cytochrome c_1. Therefore, there does not seem to be a proteolytic system in the cytosol specifically responsible for the rapid removal of excess amounts of accumulated precursor. The different stabilities of mitochondrial precursors may simply reflect differing susceptibilities of these molecules to endogenous cellular proteases.

The size differences between precursor and mature forms of the

component enzymes of OGDC fall within the range observed for mitochondrial precursor extension sequences of M_r 500-10000 (Hay et al., 1984). In general, the variability in size of mitochondrial presequences is greater than that of signal sequences of secreted proteins which consist of 15-30 amino acids. It could be argued that the larger size and greater variability of extension sequences in mitochondrial precursors is a result of the greater amount of information encoded in these sections of polypeptide chain. An example of this would be cytochrome b_2, an intermembrane space protein in yeast (Daum et al., 1982), the precursor of which is 10000 larger in M_r than the mature protein. The presequence of this protein must contain information necessary for targetting to mitochondria, and specifically to the intermembrane space, a stop transfer signal which prevents the whole protein being imported into the matrix and two specific proteolytic cleavage sites as this precursor is processed by a two-step mechanism (section 1.6.2). As previously mentioned (section 5.9), for proteins which normally exist as highly ordered multimolecular aggregates, the presequence may contain information which prevents premature aggregation of subunits in the cytosol.

In the case of multiprotein assemblies, there is also the problem of co-ordinating the expression of the individual protein subunits of the multienzyme complex. Cell lines derived from patients with Maple Syrup Urine Disease were probed with antiserum raised against BCDC using the immunoblotting technique. The results revealed that, although the E2 component of BCDC was missing from the mitochondria of one cell line, the other enzyme subunits of this complex were present (Danner et al., 1985). Therefore, lack of E2 had not interfered with synthesis of the other enzyme components of this complex and, as

mature, not precursor forms were observed it would appear that processing also was not affected. Although the E1 and E3 subunits were present in these mitochondria, presumably they are not assembled since the presence of the E2 'core' would be required before this could occur. A similar situation was observed in immunoblotting analysis, employing PDC antiserum, of fibroblasts from patients with lactic acidosis (J. A. Hodgson, personal communication). It was obvious that in some of these patients the E2 component of PDC was missing, while in others it was either E1 or component X, the associated polypeptide of unknown function (De Marcucci & Lindsay, 1985) which was absent. In all cases studied, although one enzyme component of PDC was missing the cell extracts contained the mature forms of the other subunits. Whether the PDC in these cells is assembled or not remains to be determined. In those cell lines which lack only E1 or E3 it is possible that E2-E3 or E2-E1 subcomplexes exist.

Examination of tissue samples from a child suffering from persistant metabolic acidosis and having elevated blood levels of pyruvate, 2-oxoglutarate and branched-chain 2-oxo acids revealed that all tissues were defective in both PDC and OGDC activity. Measurement of subunit specific activities revealed a deficiency in E3, explaining why both PDC and OGDC activities were absent, E3 being common to both complexes. Activity of the E1 component of PDC was normal and the subunit activities of OGDC were not tested (Robinson et al., 1977).

When BCDC is induced by differentiation of 3T3-L1 fibroblasts into adipocytes, the proteins increase disproportionately. E1 is increased almost twenty fold, E2 six fold and E3 four fold, based on

activity measurements (Chuang et al., 1983). Therefore, there is no suggestion of co-ordinate expression of the components of either PDC or BCDC.

The question of regulation of expression of E3 is interesting since this protein is involved in the structure and function of all three 2-oxo acid dehydrogenase complexes. One possible method for looking at this problem involves isolation of the genes coding for the component enzymes of these complexes. Analysis of sequences upstream from the coding regions may reveal information about control of expression. Also, addition of these cloned genes to cell lines containing mutations in one specific protein may allow the events involved in assembly of these multienzyme complexes to be studied. Indeed a cDNA clone for the transacylase component of BCDC has been identified (Litwer & Danner 1985).

6.2 PRELIMINARY STUDIES ON THE ASSEMBLY OF THE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

With the development of conditions for accumulation of precursors in the cytosol and subsequent import and processing of these proteins on removal of uncouplers, the problem of assembly was ready to be addressed. The E2 'core' of OGDC is a highly-ordered aggregate of 24 E2 subunits with a total M_r of approximately 1×10^6. It is, therefore, unlikely that such a structure would be assembled prior to import into mitochondria. The form of pre-E2 accumulated in the cytosol of cells labelled with [^{35}S]methionine in the presence of uncouplers was investigated by passing either total homogenates of cells or a post-mitochondrial fraction down various gel filtration

columns prior to immune precipitation with anti-E2 serum. Samples for gel filtration were prepared in the absence of detergents, in case the detergents altered the mobility of precursors on these columns. However, before immune precipitation, the detergent concentration in the buffer was adjusted to equal that of 3D-TKM. The main problem in these experiments was dilution of the sample. Two petri-dishes of [^{35}S]methionine-labelled cell extract was loaded onto the columns. On elution, the peak fractions contained 2-5 x 10^6 c.p.m.. In immune precipitation from detergent extracts of whole cells antisera was added to aliquots of extract containing 10-20 x 10^6 c.p.m.. This generally gave sufficient [^{35}S]methionine-labelled protein in the immune complex to allow detection after SDS-PAGE and fluorographic analysis. From 2-5 x 10^6 c.p.m., there was too little radiolabelled precursor in the products of immune precipitation to permit visualisation and, of course, pre-E2 may not be located in the peak fractions. Immune precipitates from these column fractions were also more susceptible to contamination than normal. Attempting to load more c.p.m. onto the columns initially would not only be expensive, but may create problems, such as poor resolution, associated with loading increased volumes and too much protein onto gel filtration columns.

In the literature protocols involving gel filtration prior to immune precipitation employ _in vitro_ translation products as starting material (Zimmermann & Neupert, 1980a; Miura _et al._, 1981; Ono _et al._, 1985). In this way proteins are labelled to a higher specific activity initially.

Attempts to immunoprecipitate pre-E2 from primary translation products of rat liver mRNA (a gift from G.H.D. Clarkson) failed to reveal the expected product, which probably reflects the poor cross

reactivity of antisera raised to bovine heart proteins with the corresponding enzymes from rat liver. In vitro translation of bovine heart mRNA (a gift from G.H.D. Clarkson) did not give as high incorporation of [^{35}S]methionine as rat liver mRNA. Immune precipitation was attempted from these in vitro translation products (0.5×10^6) using anti-E2 serum. After exposing the dried gel to film for one month a faint band of M_r equivalent to pre-E2 was observed. A further purification of mRNA from bovine heart was tried using the guanidinium thiocyanate procedure of Chirguin et al. (1979) to determine whether incorporation of [^{35}S]methionine into protein was improved by using freshly-made mRNA. This proved not to be so and subsequent immune precipitation from this translation was heavily contaminated with many protein bands of high and low M_r values. The same protein bands were present in immune precipitations from translation products from bovine heart mRNA and from the control, globin mRNA, suggesting a problem in the methodology. In the first immune precipitation from an in vitro translation mix, ribosomes were removed by centrifugation prior to the addition of antisera. This step was omitted in immune precipitation from the products of in vitro translation directed by the second batch of bovine heart mRNA and it now appears to be necessary to reduce the level of background in immune precipitations.

6.3 FUTURE WORK

In order to be able to carry out studies on assembly it will be necessary to purify mRNA from a suitable source and define optimal conditions for translation followed by immune precipitation. It will

also be necessary to determine maximal conditions for immune precipitation of native OGDC in order to have conditions where subunit interactions are preserved and under which the order of assembly can be examined. The 3D-TKM buffer system will be unsuitable for studies on assembly since it causes dissociation of OGDC. Possibly, in immune precipitation from in vitro translation products, less stringent conditions may be required, under which immune precipitates are free from contaminating polypeptides, but subunit interactions are preserved. Certainly the problem of nuclear lysis will not apply.

The question of cofactor addition to the component enzymes of OGDC was not addressed at all. It may be possible to study this by immune precipitation from cells labelled, in the presence of uncouplers, with a radioactive derivative of the coenzyme or prosthetic group e.g. [^{35}S]lipoic acid. If coenzyme attachment occurs inside the mitochondrion, it may be possible to examine whether it is inserted before or after processing, by immune precipitation from cells labelled with radiolabelled coenzyme, in the presence of 1,10-phenanthroline, which would allow import of the precursor into the mitochondrion but prevent processing by inhibiting the matrix protease. Metabolism of the cofactor would have to be taken into account before these types of experiments were carried out.

REFERENCES

Ades, I.Z. (1983) Biochem. Biophys. Res. Commun. 110, 42-47

Ades, I.Z. & Butow, R.A. (1980a) J. Biol. Chem. 255, 9918-9924

Ades, I.Z. & Butow, R.A. (1980b) J. Biol. Chem. 255, 9925-9935

Ades, I.Z. & Harpe, K.G. (1981) J. Biol. Chem. 256, 9329-9333

Amarasingham, C.R. & Davis, B.D. (1965) J. Biol. Chem. 240, 3664-3668

Ambrose, M.C. & Perham, R.N. (1976) Biochem. J. 155, 429-432

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. & Young, I.G. (1981) Nature 290, 457-465

Angelides, K.J. & Hammes, G.C. (1979) Biochemistry 18, 5531-5537

Argan, C., Lusty, C.J. & Shore, G.C. (1983) J. Biol. Chem. 258, 6667-6670

Arscott, L.D., Berman, M. & Williams, C.H. (1984) in Flavins & Flavoproteins (8th International Symposium on Flavins & Flavoproteins), pp. 153-156, Walter de Gruyter & Co., Berlin.

Attardi, G. & Ching, E. (1979) Methods Enzymol. 56G, 66-79

Autor, A.P. (1982) J. Biol. Chem. 257, 2713-2718

Bates, D.L., Harrison, R.A. & Perham, R.N. (1975) FEBS Lett. 60, 427-430

Batteiger, B., Newhall, W.J. & Jones, R.B. (1982) J. Immunol. Meth. 55, 297-307

Blobel, G. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 1496-1500

Blobel, G. & Dobberstein, B. (1975) J. Cell Biol. 67, 835-851

Bohni, P.C., Daum, G. & Schatz, G. (1983) J. Biol. Chem. 258, 4939-4943

Bonner, W.M. & Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88

Bosma, H.J., de Graaf-Hess, A.C., de Kok, A., Veeger, C., Visser, A.J.W.G. & Voordouw, G. (1982) Anal. N.Y. Acad. Sci. 378, 265-285

Brown, J.P. & Perham, R.N. (1976) Biochem. J. 155, 419-427

Campbell, M.T., Sutton, R. & Pollack, J.K. (1982) Eur. J. Biochem. 122, 401-406

Cerletti, N., Bohni, P.C. & Suda, K. (1983) J. Biol. Chem. 258, 4944-4949

Chamberlain, J.P. (1979) Anal. Biochem. 98, 132-135

Chance, B. & Hagihara, B. (1963) in Proc. 5th Intl. Congress of Biochemistry (Sissakian, A.N.M., ed.), vol. 5, pp. 3-37, Pergamon Press, New York

Chirguin, J.M., Przybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979) Biochemistry 18, 5294-5299

Chomyn, A., Mariottini, P., Cleeter, M.W.J., Ragan, C.I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R.F. & Attardi, G. (1985) Nature 314, 592-597

Chua, N-H. & Schmidt, G.W. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 6110-6114

Chuang, D.T., Nui, W. & Cox, R.P. (1981) Biochem. J. 200, 59-67

Chuang, D.T., Hu, C-W.C. & Patel, M.S. (1983) Biochem. J. 214, 177-181

Collins, J.H. & Reed, L.J. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 4223-4227

Conboy, J.G. & Rosenberg, L.E. (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 3073-3077

Cook, K.G., Lawson, R. & Yeaman, S.J. (1983) FEBS Lett. 164, 85-88

Côté, C., Solioz, M. & Schatz, G. (1979) J. Biol. Chem. 254, 1437-1439

Cullis, P.R. & de Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399-420

Danner, D.J., Lemmon, S.K., Besharse, J.C. & Elsas, L.J. (1979) J. Biol. Chem. 254, 5522-5526

Danner, D.J., Armstrong, N., Heffelfinger, S.C., Sewell, E.T., Priest, J.H. & Elsas, L.J. (1985) J. Clin. Invest. 75, 858-860

Danson, M.J., Hale, G., Johnson, P. & Perham, R.N. (1979) J. Mol. Biol. 129, 603-617

Date, T., Zwizinski, C., Ludmerer, S. & Wickner, W. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 827-831

Daum, G., Gasser, S.M. & Schatz, G. (1982) J. Biol. Chem. 257, 13075-13080

De Marcucci, O. & Lindsay, J.G. (1985) Eur. J. Biochem. 149, 641-648

De Marcucci, O.G.L., Clarkson, G.H.D. & Lindsay, J.G. (1985a) Biochem. Soc. Trans. 13, 347-348

De Marcucci, O.L., Hunter, A. & Lindsay, J.G. (1985b) Biochem. J. 226, 509-517

Denton, R.M. & McCormack, J.G. (1980) FEBS Lett. 119, 1-8

Denton, R.M., McCormack, J.G. & Edgell, N.J. (1980) Biochem. J. 190, 107-117

De Robertis, E.M., Longthorne, R.F. & Gurdon, J.B. (1978) Nature 272, 254-256

De Rosier, D.J., Oliver, R.M. & Reed, L.J. (1971) Proc. Nat. Acad. Sci. U.S.A. 68, 1135-1137

De Vries, H. & van't Sant, P. (1983) Horizons in Biochemistry & Biophysics 7, 249-277

Diatewa, M. & Stahl, A.J.C. (1981) Nucleic Acid Res. 9, 6293-6304

D'Souza, S.F. & Srere, P.A. (1983) J. Biol. Chem. 258, 4706-4709

Du Bois, R.N., Simpson, E.R., Tuckey, J., Lambeth, J.D. & Waterman, M.R. (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 1028-1032

Felipo, V., Miralles, V., Knecht, E., Hernandez-Yago, J. & Grisolia, S. (1983) Eur. J. Biochem. 133, 641-644

Fenton, W.A., Hack, A.M., Helfgott, D. & Rosenberg, L.E. (1984) J. Biol. Chem. 259, 6616-6621

Freitag, H., Neupert, W. & Benz, R. (1982a) Eur. J. Biochem. 123, 629-636

Freitag, H., Janes, M. & Neupert, W. (1982b) Eur. J. Biochem. 126, 197-202

Garland, P.B. (1964) Biochem. J. 92, 10C-12C

Gasser, S.M. (1983) Methods Enzymol. 97, 329-336

Gasser, S.M. & Schatz, G. (1983) J. Biol. Chem. 258, 3427-3430

Gasser, S.M., Daum, G. & Schatz, G. (1982a) J. Biol. Chem. 257, 13034-13041

Gasser, S.M., Ohashi, A., Daum, G., Bohni, P.C., Gibson, J., Reid, G.A., Yonetani, T. & Schatz, G. (1982b) Proc. Nat. Acad. Sci. U.S.A. 79, 267-271

Gilbert, G.A. & Gilbert, L.M. (1980) J. Mol. Biol. 144, 405-408

Giles, N.H., Partridge, C.W.H., Ahmed, S.I. & Case, M.E. (1967) Proc. Nat. Acad. Sci. U.S.A. 58, 1930-1937

Goldman, B.M. & Blobel, G. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 5066-5070

Gorringe, D.M. & Moses, V. (1978) Biochem. Soc. Trans. 6, 167-169

Guest, J.R. (1974) J. Gen. Microbiol. 80, 523-532

Guest, J.R. & Creaghan, I.T. (1973) J. Gen. Microbiol. 75, 197-210

Hackert, M.L., Oliver, R.M. & Reed, L.J. (1983) Proc. Nat. Acad. Sci. U.S.A. 80, 2226-2230

Hallermeyer, G., Zimmermann, R. & Neupert, W. (1977) Eur. J. Biochem. 81, 523-532

Hampsey, D.M., Lewin, S. & Kohlhaw, G.B. (1983) Proc. Nat. Acad. Sci. U.S.A. 80, 1270-1274

Hansen, R.G. & Henning, U. (1966) Biochim. Biophys. Acta 122, 355-358

Hansford, R.G. (1972) FEBS Lett. 21, 139-141

Harmey, M.A. & Neupert, W. (1979) FEBS Lett. 108, 385-389

Harmey, M.A. & Neupert, W. (1984) Biochem. Soc. Trans. 12, 373-374

Hase, T., Riezman, H., Suda, K. & Schatz, G. (1983) EMBO J. 2, 2169-2172

Hase, T., Muller, U., Riezman, H. & Schatz, G. (1984) EMBO J. 3, 3157-3164

Hatalova, I. & Kolarov, J. (1983) Biochem. Biophys. Res. Commun. 110, 132-139

Hay, R., Bohni, P. & Gasser, S.M. (1984) Biochim. Biophys. Acta 779, 65-87

Henderson, C.E. & Perham, R.N. (1980) Biochem. J. 189, 161-172

Hennig, B. & Neupert, W. (1981) Eur. J. Biochem. 121, 203-212

Hennig, B. & Neupert, W. (1983) Horizons in Biochemistry & Biophysics 7, 307-346

Hennig, B., Koehler, H. & Neupert, W. (1983) Proc. Nat. Acad. Sci. U.S.A. 80, 4963-4967

Highfield, P.E. & Ellis, R.J. (1978) Nature 271, 420-424

Hirabayashi, T. & Harada, T. (1971) Biochem. Biophys. Res. Commun. 45, 1369-1375

Hirashima, M., Hayakawa, T. & Koike, M. (1967) J. Biol. Chem. 242, 902-907

Hodgson, J.A. (1983) PhD Thesis, University of Cambridge

Hodgson, J.A., Lowe, P.N. & Perham, R.N. (1983) Biochem. J. 211, 463-472

Horwich, A.L., Kalousek, F., Mellman, I. & Rosenberg, L.E. (1985a) EMBO J. 4, 1129-1135

Horwich, A.L., Fenton, W.A., Firgaira, F.A., Fox, J.E., Kolansky, D., Mellman, I.S. & Rosenberg, L.E. (1985b) J. Cell Biol. 100, 1515-1521

Hurt, E., Pesold-Hurt, B. & Schatz, G. (1984a) EMBO J. 3, 3149-3156

Hurt, E., Pesold-Hurt, B. & Schatz, G. (1984b) FEBS Lett. 178, 306-310

Ishikawa, E., Oliver, R.M. & Reed, L.J. (1966) Proc. Nat. Acad. Sci. U.S.A. 56, 534-541

Jackson, R.C. & Blobel, G. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 5598-5602

Jackson, R.H. & Singer, T.P. (1983) J. Biol. Chem. 258, 1857-1865

Jaussi, R., Sonderegger, P., Flückiger, J. & Christen, P. (1982) J. Biol. Chem. 257, 13334-13340

Jeyaseelan, K., Guest, J.R. & Visser, J. (1980) J. Gen. Microbiol. 120, 393-402

Kalousek, F., Orsulak, M.D. & Rosenberg, L.E. (1984) J. Biol. Chem. 259, 5392-5395

Kanzaki, T., Hayakawa, T., Hamada, M., Fukuyoshi, Y. & Koike, M. (1969) J. Biol. Chem. 244, 1183-1187

Kaput, J., Goltz, S. & Blobel, G. (1982) J. Biol. Chem. 257, 15054-15058

Kellems, R.E., Allison, V.F. & Butow, R.A. (1975) J. Cell Biol. 65, 1-14

Kreibich, G., Ulrich, B.L. & Sabatini, D.D. (1978a) J. Cell Biol. 77, 464-487

Kreibich, G., Freienstein, C.M., Pereyra, B.N., Ulrich, B.L. & Sabatini, D.D. (1978b) J. Cell Biol. 77, 488-506

Koike, M. & Koike, K. (1976) Adv. Biophys. 9, 187-227

Koike, M. & Koike, K. (1982) Anal. N.Y. Acad. Sci. 378, 225-235

Koike, M., Reed, L.J. & Carroll, W.R. (1960) J. Biol. Chem. 235, 1924-1930

Koike, K., Hamada, M., Tanaka, N., Otsuka, K-I., Ogasahara, K. & Koike, M. (1974) J. Biol. Chem. 249, 3836-3842

Korb, H. & Neupert, W. (1978) Eur. J. Biochem. 91, 609-620

Kornfeld, S., Benziman, M. & Milner, Y. (1977) J. Biol. Chem. 252, 2940-2947

Kresze, G-B. & Steber, L. (1979) Eur. J. Biochem. 95, 569-572

Kresze, G-B. & Ronft, H. (1981a) Eur. J. Biochem. 119, 573-579

Kresze, G-B. & Ronft, H. (1981b) Eur. J. Biochem. 119, 581-587

Kresze, G-B., Ronft, H., Dietl, B. & Steber, L. (1981) FEBS Lett. 127, 157-160

Laemmli, U.K. (1970) Nature 227, 680-685

Laskey, R.A. & Mills, A.D. (1977) FEBS Lett. 82, 314-316

Lawson, R., Cook, K.G. & Yeaman, S.J. (1983) FEBS Lett. 157, 54-58

LaPolla, R.J. & Lambowitz, A.M. (1981) J. Biol. Chem. 256, 7064-7067

Lewin, A.S. & Norman, D.K. (1983) J. Biol. Chem. 258, 6750-6755

Lewin, A.S., Gregor, I., Mason, T.L., Nelson, N. & Schatz, G. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 3998-4002

Linn, T.C. (1971) Arch. Biochem. Biophys. 161, 505-514

Linn, T.C., Pelley, J.W., Pettit, F.H., Hucho, F., Randall, D.D. & Reed, L.J. (1972) Arch. Biochem. Biophys. 148, 327-342

Litwer, S. & Danner, D.J. (1985) Biochim. Biophys. Acta in press

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Lumsden, J. & Coggins, J.R. (1977) Biochem. J. 161, 599-607

Lustig, A., Levens, D. & Rabinowitz, M. (1982) J. Biol. Chem. 257, 5800-5808

Lynen, F. (1972) in Proc. 8th FEBS Meeting, pp. 177-200, Elsevier, N. Holland

Lynen, A., Sedlaczek, E. & Wieland, O.H. (1978) Biochem. J. 169, 321-328

Maccecchini, M-L., Rudin, Y., Blobel, G. & Schatz, G. (1979a) Proc. Nat. Acad. Sci. U.S.A. 76, 343-347

Maccecchini, M-L., Rudin, Y. & Schatz, G. (1979b) J. Biol. Chem. 254, 7468-7471

Markwell, M.A.K., Haas, S.M., Bieber, L.L. & Tolbert, N.E. (1978) Anal. Biochem. 87, 206-210

Massey, V. (1960) Biochim. Biophys. Acta 38, 447-460

Massey, V. (1963) The Enzymes, 2nd edn. (Boyer, P.D., Lardy, H. & Myrback, K., ed.), vol. 7, pp. 275-306, Academic Press Inc. (London) Ltd.

Massey, V. & Veeger, C. (1961) Biochim. Biophys. Acta 48, 33-47

Massey, V., Gibson, Q.H. & Veeger, C. (1960) Biochem. J. 77, 341-351

Matsuura, S., Arpin, M., Hannum, C., Margoliash, E., Sabatini, D.D. & Morimoto, T. (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 4368-4372

Matuda, S. & Saheki, T. (1982) J. Biochem. 91, 553-561

Matuda, S., Shirahama, T., Saheki, T., Miura, S. & Mori, M. (1983) Biochim. Biophys. Acta 741, 86-93

McAda, P.C. & Douglas, M.G. (1982) J. Biol. Chem. 257, 3177-3182

McCormack, J.G. & Denton, R.M. (1979) Biochem. J. 180, 533-544

McCormack, J.G. & Denton, R.M. (1981) Biochem. J. 196, 619-624

Meyer, D.I., Louvard, D. & Dobberstein, B. (1982a) J. Cell Biol. 92, 579-583

Meyer, D.I., Krause, E. & Dobberstein, B. (1982b) Nature 297, 647-650

Michael, R., Wachter, E. & Sebald, W. (1979) FEBS Lett. 101, 373-376

Mihara, K. & Blobel, G. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 4160-4164

Mihara, K. & Sato, R. (1985) EMBO J. 4, 769-774

Mihara, K., Blobel, G. & Sato, R. (1982a) Proc. Nat. Acad. Sci. U.S.A. 79, 7102-7106

Mihara, K., Omura, T., Harano, T., Brenner, S., Fleischer, S., Rajagopalan, K.V. & Blobel, G. (1982b) J. Biol. Chem. 257, 3355-3358

Milstein, C., Brownlee, G.G., Harrison, T.M. and Matthews, M.B. (1972) Nature New Biol. 239, 117-120

Miura, S., Mori, M., Amaya, Y., Tatibana, M. & Cohen, P.P. (1981) Biochem. Int. 3, 305-312

Miura, S., Mori, M. & Tatibana, M. (1983) J. Biol. Chem. 258, 6671-6674

Miyazawa, S., Ozasa, H., Furuta, S., Osumi, T., Hashimoto, T., Miura, S., Mori, M. & Tatibana, M. (1983) J. Biochem. 93, 453-457

Mori, M., Miura, S., Tatibana, M. & Cohen, P.P. (1979) J. Biol. Chem. 254, 3141-3144

Mori, M., Miura, S., Tatibana, M. & Cohen, P.P. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 7044-7048

Mori, M., Morita, T., Ikeda, F., Amaya, Y., Tatibana, M. & Cohen, P.P. (1981a) Proc. Nat. Acad. Sci. U.S.A. 78, 6056-6060

Mori, M., Morita, T., Miura, S. & Tatibana, M. (1981b) J. Biol. Chem. 256, 8263-8266

Morita, T., Mori, M., Tatibana, M. & Cohen, P.P. (1981) Biochem. Biophys. Res. Commun. 99, 623-629

Morita, T., Miura, S., Mori, M. & Tatibana, M. (1982) Eur. J. Biochem, 122, 501-509

Mosmann, T.R., Bowmal, R. & Williamson, A.R. (1979) Eur. J. Immunol. 9, 511-516

Muekler, M.M., Himeno, M. & Pitot, H.C. (1982) J. Biol. Chem. 257, 7178-7180

Murphy, E., Coll, K., Rich, T.L. & Williamson, J.R. (1980) J. Biol. Chem. 255, 6600-6608

Nabi, N. & Omura, T. (1980) Biochem. Biophys. Res. Commun. 97, 680-686

Nawa, H., Brady, W.R., Koike, M. & Reed, L.J. (1960) J. Am. Chem. Soc. 82, 896-903

Nelson, N. & Schatz, G. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 4365-4369

Neupert, W. & Schatz, G. (1981) Trends Biochem. Sci. 6, 1-4

Ohashi, A., Gibson, J., Gregor, I. & Schatz, G. (1982) J. Biol. Chem. 257, 13042-13047

Ohne, M. (1975) J. Bacteriol. 122, 224-234

Ohta, S. & Schatz, G. (1984) EMBO J. 3, 651-657

Oliver, R.M. & Reed, L.J. (1982) in Electron Microscopy of Proteins (Harris, J.R., ed.), vol. 2, pp. 1-48, Academic Press Inc. (London) Ltd.

Ono, H. & Tuboi, S. (1985) Biochem. Int. 10, 351-357

Ono, H., Yoshimura, N., Sato, M. & Tuboi, S. (1985) J. Biol. Chem. 260, 3402-3407

Parker, M.G. & Weitzman, P.D.J. (1973) Biochem. J. 135, 215-223

Passarella, S., Marra, E., Doonan, S., Saccone, C. & Quagliariello, E. (1984) Biochem. Soc. Trans. 12, 381-382

Perham, R.N. (1975) Philos. Trans. Royal Soc. London, Ser B, 272, 123-136

Perham, R.N. & Thomas, J.O. (1971) FEBS Lett. 15, 8-12

Perham, R.N. & Roberts, G.C.K. (1981) Biochem. J. 199, 733-740

Perham, R.N., Duckworth, H.W. & Roberts, G.C.K. (1981) Nature 292, 474-477

Perryman, M.B., Strauss, A.N., Olson, J. & Roberts, R. (1983) Biochem. Biophys. Res. Commun. 110, 967-972

Pettit, F.H., Hamilton, L., Munk, P., Namihira, G., Eley, M., Willms, C.R. & Reed, L.J. (1973) J. Biol. Chem. 248, 5282-5290

Pettit, F.H., Yeaman, S.J. & Reed L.J. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 4881-4885

Poyton, R. & McKemmie, E. (1979) J. Biol. Chem. 254, 6763-6771

Racker, E. (1950) Biochim. Biophys. Acta 4, 211-214

Raymond, Y. & Shore, G.C. (1979) J. Biol. Chem. 254, 9335-9338

Raymond, Y. & Shore, G.C. (1981) J. Biol. Chem. 256, 2087-2090

Reed, L.J. (1974) Acc. Chem. Res. 7, 40-46

Reed, L.J. & Oliver, R.M. (1968) Brookhaven Symp. Biol. 21, 397-412

Reed, L.J. & Mukherjee, B.B. (1969) Methods Enzymol. 13, 55-61

Reed, L.J. & Oliver, R.M. (1982) Adv. Exp. Med. Biol. 148, 231-241

Reed, L.J. & Yeaman, S.J. (1985) The Enzymes, 3rd edn. (Boyer, P.D., ed.), in press, Academic Press Inc. (London) Ltd.

Reid, G.A. & Schatz, G. (1982) J. Biol. Chem. 257, 13062-13067

Reid, G.A., Yonetani, T. & Schatz, G. (1982) J. Biol. Chem. 257, 13068-13074

Roberts, C.K., Duckworth, H.W., Packman, L.C. & Perham, R.N. (1982) Ciba Found. Symp. 93, 47-71

Robinson, B.H., Taylor, J. & Sherwood, W.G. (1977) Pediat. Res. 11, 1198-1202

Roche, T.E. & Cate, R.L. (1977) Arch. Biochem. Biophys. 183, 664-677

Roche, T.E. & Lawlis, V.B. (1982) Anal. N.Y. Acad. Sci. 378, 236-249

Riezman, H., Hay, R., Gasser, S.M., Daum, G., Schneider, G., Witte, C. & Schatz, G. (1983a) EMBO J. 2, 1105-1111

Riezman, H., Hay, R., Witte, C., Nelson, N. & Schatz, G. (1983b) EMBO J. 2, 1112-1118

Riezman, H., Hase, T., van Loon, A.P.G.M., Grivell, L.A., Suda, K. & Schatz, G. (1983c) EMBO J. 2, 2161-2168

Sabatini, D.D., Kreibich, G., Morimoto, T. & Adesnik, M. (1982) J. Cell Biol. 92, 1-22

Sagara, Y. & Ito, A. (1982) Biochem. Biophys. Res. Commun. 109, 1102-1107

Sakakibara, R., Huynh, Q.K., Nishida, Y., Watanabe, T. & Wada, H. (1980) Biochem. Biophys. Res. Commun. 95, 1781-1788

Sakakibara, R., Kamisaki, Y., Horio, Y. & Wada, H. (1983) Biochem. Int. 6, 231-238

Sakurai, Y., Fukuyoshi, Y., Hamada, M., Hayakawa, T. & Koike, M. (1970) J. Biol. Chem. 245, 4453-4462

Salacinski, P.R.P., McLean, C., Sykes, J.E.C., Clement-Jones, V.V. & Lowry P.J. (1981) Anal. Biochem. 117, 136-146

Schatz, G. & Mason, T.L. (1974) Ann. Rev. Biochem. 43, 51-87

Schekman, R. (1985) Trends Biochem. Sci. 10, 177

Schieber, G.L. & O'Brien T.W. (1985) J. Biol. Chem. 260, 6367-6372

Schleyer, M., Schmidt, B. & Neupert, W. (1982) Eur. J. Biochem. 125, 109-116

Schmelzer, E. & Heinrich, P.C. (1980) J. Biol. Chem. 255, 7503-7506

Schmidt, B. & Neupert, W. (1984) Biochem. Soc. Trans. 12, 920-921

Schmidt, B., Hennig, B., Kohler, H. & Neupert, W. (1983a) J. Biol. Chem. 258, 4687-4689

Schmidt, B., Hennig, B., Zimmermann, R. & Neupert, W. (1983b) J. Cell Biol. 96, 248-255

Schrier, P.H., Seftor, E.A., Schell, J. & Bohnert, H.J. (1985) EMBO J. 4, 25-32

Shore, G.C., Power, F., Bendayan, M. & Carignan, P. (1981) J. Biol. Chem. 256, 8761-8766

Smith, A.L. (1967) Methods Enzymol. 10, 81-86

Smith, C.M., Bryla, J. & Williamson, J.R. (1974) J. Biol. Chem. 249, 1497-1505

Sokatch, J.R., McCully, V., Gebrosky, J. & Sokatch, D.J. (1981a) J. Bacteriol. 148, 639-646

Sokatch, J.R., McCully, V. & Roberts, C.M. (1981b) J. Bacteriol. 148, 647-652

Sonderegger, P., Jaussi, R. & Christen, P. (1980) Biochem. Biophys. Res. Commun. 94, 1256-1260

Sonderegger, P., Jaussi, R., Christen, P. & Gehring, H. (1982) J. Biol. Chem. 257, 3339-3345

Srere, P.A. (1969) Methods Enzymol. 13, 3-11

Stanley, C.J. & Perham, R.N. (1980) Biochem. J. 191, 147-154

Stephens, P.E., Lewis, H.M., Darlison, M.G. & Guest, J.R. (1983) Eur. J. Biochem. 135, 519-539

Stepp, L.R., Bleile, D.M., McRorie, D.K., Pettit, F.H. & Reed, L.J. (1981) Biochemistry 20, 4555-4560

Suissa, M. & Schatz, G. (1982) J. Biol. Chem. 257, 13048-13055

Sumegi, B. & Srere, P.A. (1984) J. Biol. Chem. 259, 15040-15045

Takiguchi, M., Miura, S., Mori, M. & Tatibana, M. (1983) Comp. Biochem. Physiol. 75B, 227-231

Tanaka, N., Koike, K., Hamada, M., Otsuka, K-I., Suematsu, T. & Koike, M. (1972) J. Biol. Chem. 247, 4043-4049

Tanaka, N., Koike, K., Otsuka, K-I., Hamada, M., Ogasahara, K. & Koike, M. (1974) J. Biol. Chem. 249, 191-198

Tasheva, B. & Dessev, G. (1983) Anal. Biochem. 129, 98-102

Teintze, M., Slaughter, M., Weiss, H. & Neupert, W. (1982) J. Biol. Chem. 257,10364-10371

Terpstra, P. & Butow, R.A. (1979) 254, 12662-12669

Todd, P.E.E., East, I.J. & Leach, S.J. (1982) Trends Biochem. Sci. 7, 212-216

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 4350-4354

Van den Broeck, G., Timko, M.P., Kausch, A.P., Cashmore, A.R., van Montagu, M. & Herrera-Estrella, L. (1985) Nature 313, 358-363

Viebrock, A., Perz, A. & Sebald, W. (1982) EMBO J. 1, 565-571

Visser, J., Kester, H. & Huigen, A. (1980) FEMS Microbiol. Lett. 9, 227-232

Walter, P. & Blobel, G. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 7112-7116

Walter, P. & Blobel, G. (1982) Nature 299, 691-698

Walter, P., Ibrahimi, I. & Blobel, G. (1981) J. Cell Biol. 91, 545-550

Warwrzynczak, E.J., Perham, R.N. & Roberts, G.C.K. (1981) FEBS Lett. 131, 151-154

Watanabe, K. & Kubo, S. (1982) Eur. J. Biochem. 123, 587-592

Wedding, R.T. & Black, M.K. (1971) J. Biol. Chem. 246, 1638-1643

Weiland, O.H. (1975) FEBS Lett. 52, 44-47

Westhof, E., Altschuh, D., Moras, D., Bloomer, A.C., Mondragon, A., Klug, A. & van Regenmortel M.H.V. (1984) Nature 311, 123-126

Williamson, J.R. & Cooper, R.H. (1980) FEBS Lett. 117, K73-K84

White, R.H., Bleile, D.M. & Reed L.J. (1980) Biochem. Biophys. Res. Commun. 94, 78-84

Yaffe, M.P. & Schatz, G. (1984) Proc. Nat. Acad. Sci. U.S.A. 81, 4819-4823

Yanofsky, C & Crawford, I.P. (1972) The Enzymes, 3rd edn. (Boyer, P.D., ed.), vol. 7, pp. 1-31, Academic Press Inc. (London) Ltd.

Yamauchi, K., Hayashi, N. & Kikuchi, G. (1980) FEBS Lett. 115, 15-18

Zimmermann, R. & Neupert, W. (1980a) Eur. J. Biochem. 109, 217-229

Zimmermann, R. & Neupert, W. (1980b) Eur. J. Biochem. 112, 225-233

Zimmermann, R., Paluch, U. & Neupert, W. (1979a) FEBS Lett. 108, 141-146

Zimmermann, R., Paluch, U., Sprinzl, M. & Neupert, W. (1979b) Eur. J. Biochem. 99, 247-252

Zimmermann, R., Hennig, B. & Neupert, W. (1981) Eur. J. Biochem. 116,

Zimmermann, R., Hennig, B. & Neupert, W. (1981) Eur. J. Biochem. 116, 455-460

Zuurendonk, P.F. & Tager, J.M. (1974) Biochim. Biophys. Acta 333, 393-399

Zwizinski, C., Schleyer, M. & Neupert, W. (1983) J. Biol. Chem. 258, 4071-4074

Zwizinski, C., Schleyer, M. & Neupert, W. (1984) J. Biol. Chem. 259, 7850-7856